EFFECTS OF OVARIAN STIMULATION ON OOCYTE DEVELOPMENT AND EMBRYO QUALITY

By

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DECLARATION

I hereby declare that the work in this thesis has been devised, conducted and composed by myself and has not been submitted for any other degree, in Nottingham or elsewhere. The work presented herein is my own, where other sources have been used, the authors have been duly acknowledged.

Kimberley Marie Swann

ABSTRACT

Ovarian stimulation plays a pivotal role in assisted reproductive therapies, to increase the number of embryos available for treatment; however, there is no clear consensus from meta-analyses in the literature which, if any, of the preparations in use are superior in terms of clinical outcomes. The aim of this thesis was to examine the effect of common human gonadotrophin preparations with different half lives and LH activity (hMG, rFSH and Pergoveris) on embryo quality and resulting offspring, compared to non-stimulated negative controls and positive PMSG treated controls, using the mouse model. The studies in this thesis indicated that an LH ceiling threshold is evident during folliculogenesis, where the use of long acting LH preparations resulted in higher numbers of fragmented oocytes, absent of cumulus cells ($P<0.001$), reduced expression of the pro and anti-angiogenic factors, *MYHII* and *PEDF* in cumulus cells ($P<0.05$), increased embryonic developmental arrest ($P<0.001$) and perturbed *IGF2* ($P<0.05$) and *VEGFA* gene expression in resulting blastocysts ($P<0.01$), compared to negative controls. Use of preparations containing LH bioactivity resulted in offspring with altered total body weight trajectories and internal organ weight abnormalities ($P<0.05$), which were, in some instances, compounded by *in vitro* culture. In addition, we elucidated a relationship between FSH half life differences between urinary and recombinant preparations and embryo quality. The urinary human gonadotrophin preparation, hMG, could yield developmentally competent embryos at lower concentrations, than the recombinant Pergoveris treatment. In addition to FSH, these preparations contain LH and both low doses of preparations composed of short half life rFSH and rLH and high doses of preparations containing long acting LH bioactivity, resulted in the highest rates of developmental arrest. These groups were observed to have complete absence of *H19* expression. The results of this thesis provide clear evidence that ovarian stimulation does negatively impact the embryo and subsequent offspring and provides support for an LH ceiling threshold, above which detrimental effects occur, both on *in vitro* embryo development and *in vivo* foetal development, which later effects postnatal growth.

PUBLICATIONS

The relationship between luteinising hormone in ovarian stimulation and subsequent embryo development and viability

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LIST OF ABBREVIATIONS

20α-HSD	20α hydroxysteroid dehydrogenase
ACE	Angiotensin Converting Enzyme
AFC	Antral Follicle Count
Ang	Angiotensin
AngII	Angiotensin II
ART	Assisted Reproductive Therapies
AS	Angelman Syndrome
AT	Angiotensin
ATR	Angiotensin Receptor
BMP	Bone Morphogenetic Proteins
BMSU	Biomedical Sciences Unit
BrdU	BromodeoxyUridine
BSA	Bovine Serum albumin
BWS	Beckwith Weidmann Syndrome
cAMP	cyclic Adenosine Mono Phosphate
cDNA	complementary DNA
CG	Chorionic Gonadotrophin
CHO	Chinese Hamster Ovary
CL	Corpus Luteum
CO_2	Carbon di Oxide
COC	Cumulus Oocyte Complex
COS	Controlled Ovarian Stimulation
CX43	Connexin 43
DAPI	4',6-diamidino-2-phenylindole

DMR	differentially methylated region
DNA	Deoxy-Ribose Nucleic Acid
Dnmt	DNA methyl transferase
DOHaD	Developmental Origins of Health and Disease
dpc	days post ciotum
ECM	Extra Cellular Matrix
eNOS	endothelial Nitric Oxide Synthase
eSEM	environmental Scanning Electron Micrography
ESHRE	European Society for Human Reproduction and Embryology
ET	Embryo transfer
EVT	Extra Villous Trophoblast
FSH	Follicle Stimulating Hormone
GDF	Growth Differentiation Factor
GnRH	Gonadotrophin Releasing Hormone
GnSAF	Gonadotrophin Surge Attenuating Factor
GV	Germinal vesicle
HAS2	Hyaluronan synthase 2
hCG	human Chorionic Gonadotrophin
HFEA	Human Fertilisation and Embryology Authority
hMG	human Menopausal Gonadotrophin
HSA	Human Serum Albumin
ICM	Inner Cell Mass
ICR	Imprinting Control Region
ICSI	Intra Cytoplasmic Sperm Injection
IGF2	Insulin Like Growth Factor
IGF2r	Insulin Like Growth Factor receptor
IGN	Imprinted Gene Network
IUGR	Intra Uterine Growth Restriction

IUI	Intra Uterine Insemination
IVF	*In Vitro* Fertilisation
KO	knockout
LH	Luteinising Hormone
LOS	Large Offspring Syndrome
MeCP	methyl CpG binding protein
MI	Metaphase I
MII	Metaphase II
MMP	Matrix Metalloproteinase
mRNA	messenger Ribosomal Nucleic Acid
MYHII	Myosin Heavy chain II
NSET	None Surgical Embryo Transfer
OHSS	Ovarian Hyper Stimulation Syndrome
P_4	Progesterone
P450c17	cytochrome P450 enzyme 17 alpha hydroxylase
PBS	Phospate Buffered Saline
PCR	Polymerase chain reaction
PEDF	Pigment Epithelium Derived Factor
PFA	Para Form Aldehyde
PGC	Primordial Germ Cell
PGD	Preimplantation Genetic Diagnosis
PGS	Preimplantation Genetic Screening
PI	Propidium Iodide
PMSG	Pregnant Mare Serum Gonadotrophin
RAS	Renin Angiotensin System
rFSH	recombinant Follicle Stimulating Hormone
rhCG	recombinant human Chorionic Gonadotrophin
rLH	recombinant Luteinising Hormone

RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SEM	Scanning Electron Micrography
serpin	serine protease inhibitors
SRS	Silver Russell Syndrome
TCN	Total Cell Number
TdT	Terminal deoxynucleotidyl Transferase
TE	Trophectoderm
TGC	Trophoblast Giant Cell
TGFb	Transforming Growth Factor beta
TNF-α	Tumour Necrosis Factor alpha
uFSH	urinary Follicle Stimulating Hormone
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organisation
WOW	Well of the Well
ZGA	Zygotic Gene Activation

CHAPTER 1: LITERATURE REVIEW

1.1 THE IMPORTANCE OF OVARIAN STIMULATION

The use of controlled ovarian stimulation (COS) plays a pivotal role in assisted reproductive therapies (ART) to increase the number of embryos available for treatment of couples unable to conceive by conventional means. Infertility is defined as the inability of a couple to spontaneously conceive following one year of regular unprotected sexual intercourse (Frey and Patel, 2004). Infertility is escalating at an unprecedented rate with a current estimate of 1 in 6 UK couples encountering problems conceiving (Kamel, 2010). A wide variety of options are now available for the clinical management of infertility. Routine treatments include intra-uterine insemination (IUI), *in vitro* fertilisation (IVF) and intra-cytoplasmic sperm injection (ICSI) and more recently pre-implantation genetic diagnosis/screening (PGD/PGS). The number of ART procedures annually undertaken is increasing with an uptake of 5.8% from 2006-2007 (HFEA, 2009). Thus it is imperative to continually refine and advance techniques such as the super-ovulatory regimes employed to improve the outcomes of treatment.

The first successful IVF birth resulted from a single oocyte aspirated during a natural menstrual cycle, soon after the luteinising hormone (LH) surge. Following the transfer of 32 cleavage stage embryos to recipient mothers, only four patients achieved pregnancy, of which three miscarried. One went to full term to become Louise Brown (Edwards *et al.*, 1980). It was logically deduced, therefore, that stimulating the ovaries would enable a greater number of mature follicles from the developing cohort to be retrieved, permitting several embryos to be transferred in order to increase success rates (Wood *et al.*, 1985). Edwards and Steptoe had considered this and originally used human menopausal gonadotrophin (hMG) and human chorionic gonadotrophin (hCG) in a COS regime discussed in more detail in section 1.6.1. However, after repeated failures

they reverted back to natural cycle IVF under the assumption that the observed shortening of the luteal phase of stimulated women may be having an adverse effect on the pregnancy rates (Beall and DeCherney, 2012). Superovulation protocols, whereby exogenous gonadotrophins are utilised to promote follicle growth whilst LH or hCG are administered for the final follicular maturation and rupture, were refined by Carl Wood and Alan Trounson in Melbourne Australia during the late 1970's and the first baby born to a patient following ovarian stimulation, Candice Reed, was born in Canada in 1980 (Challoner, 1999; Martín-Coello *et al.*, 2008).

Despite the benefit of superovulation significantly increasing the oocyte yield per cycle, it is not without its disadvantages. In mice, superovulation with PMSG has been associated with delayed embryonic development to blastocyst *in vitro* and *in vivo,* abnormal pre-implantation development, a 40% reduction in foetal growth and increased numbers of re-sorption sites (Van der Auwera and D'Hooghe, 2001). Adverse effects on embryo viability were exacerbated when higher doses of PMSG were used, possibly due to nutritional deficiency as a consequence of overcrowded uteri (Edgar *et al.*, 1987; Evans *et al.*, 1981). PMSG is no longer used in patient treatment and the few studies on the effects of human gonadotrophins on embryo development in mice are contradictory, due to strain and method variances (Brooke *et al.*, 2007; Calongos *et al.*, 2008; Muñoz *et al.*, 1995; Edirisinghe *et al.*, 1986). The effects of COS on human embryos are sparse due to ethical and moral limitations, however there are reports suggesting human singleton conceptions generated through IVF display more peri-natal complications than their naturally conceived counterparts (Jackson *et al.*, 2004; Helmerhorst *et al.*, 2004; Basatemur and Sutcliffe, 2008). The putative origins of the negative effects reported after COS could potentially occur at multiple stages during folliculogenesis and may alter the uterine environment and endometrial receptivity, which consequently may affect foetal development.

In this chapter I will provide an overview of normal physiology in mice and humans; covering oogenesis and folliculogenesis, embryo development, implantation and foetal development, before introducing the rationale for ovarian stimulation, the history behind the current preparations in clinical use and a summary of previously published findings on the negative consequences of ovarian stimulation. The literature review will provide the background for the following experiments which assess the effect of human gonadotrophin preparations on oocyte and embryo quality, genetics of cumulus and trophectoderm cells from blastocysts and ultimately postnatal growth of offspring, in comparison to non-stimulated and PMSG treated controls.

1.2 OOGENESIS AND FOLLICULOGENESIS

1.2.1 Primordial follicle formation

Oogenesis begins with the formation of primordial germ cells (PGCs) from the proximal epiblast from day 8 in mice and is thought to be controlled by the bone morphogenetic proteins; BMP2, BMP4 and BMP8b signalling from the extra-embryonic tissue (Oktem and Urman, 2010). These pluripotent cells chemotactically home to the future gonads and after colonisation is complete at approximately 9.5-11.5 days post coitum (dpc) the PGCs differentiate into oogonia, undergoing several rounds of mitotic divisions before entering meiosis and arresting at the diplotene phase of prophase I (Richards and Pangas, 2010). In mice, formation of these primordial follicles is thought to occur during the first few days post partum and are fully formed by day 3 (McGee and Hsueh, 2000). The female gametes are then stored in a quiescent state until sequentially recruited to enter the growing follicle pool (Oktem and Urman, 2010). The first wave of follicles to reach the antral stage occurs by week 3 post partum in the mouse (McGee and Hsueh, 2000).

Follicular endowment, in the human, commences pre-natally and it is a widely accepted doctrine in reproductive biology that females at birth are provisioned with the entire complement of oocytes to last their

reproductive life span (Zuckerman, 1951). PGCs are estimated to form between the third and fourth week of human gestation and reside within the future gonads by seven weeks. Primordial follicles are estimated to form around mid-gestation (Fulton *et al.*, 2005). Unlike mice, the female gametes in humans are suggested to be recruited to enter the growing follicle pool from as early as 15-16 weeks gestation. This continues postnatally until the ovarian follicular reserve is depleted (Knight and Glister, 2006). In contrast however, it has recently been proposed that new primordial follicles can form in the ovaries throughout early life in the mouse and the putative origin of these germ cells is the bone marrow (Johnson *et al.*, 2004; Johnson *et al.*, 2005). Implications of this observation remain to be determined but women still run out of oocytes.

1.2.2 Gonadotrophin independent follicles

Follicular development has three distinct phases classified according to the morphological appearance of the follicle and dependence on pituitary gonadotrophins for successive growth. The first stage, the gonadotrophin independent stage is where irreversible recruitment from the resting primordial follicles is initiated. Diplotene oocytes within primordial follicles are encased in flattened pre-granulosa cells that undergo a squamous-to-cuboidal transition to form granulosa cells. The zona pellucida is also acquired during this transition from primordial to the primary follicle stage, the earliest form of a committed follicle (Oktem and Urman, 2010).

As follicles further increase in size and develop into secondary and then tertiary follicles, the single granulosa cell layer becomes multi-layered through mitotic expansion and the basal lamina and theca cell layer are formed from differentiation of the surrounding stromal cells (Knight and Glister, 2006). This incessant phase of folliculogenesis initiates 15-16 weeks into gestation and continues throughout post-natal development and adulthood until the ovary is depleted of its stockpile and the woman enters the menopause. These early gonadotrophin independent stages of follicle growth are controlled by the co-ordinated and synergistic actions of an extensive array of cytokines and growth factors from various signal

transduction pathways including the transforming growth factor beta (TGFβ) super-family as summarised in Figure 1.1 (Oktem and Urman, 2010; Knight and Glister, 2006). Ovarian stimulation is assumed to have no effect on these early stages of folliculogenesis and therefore they will not be discussed in further detail.

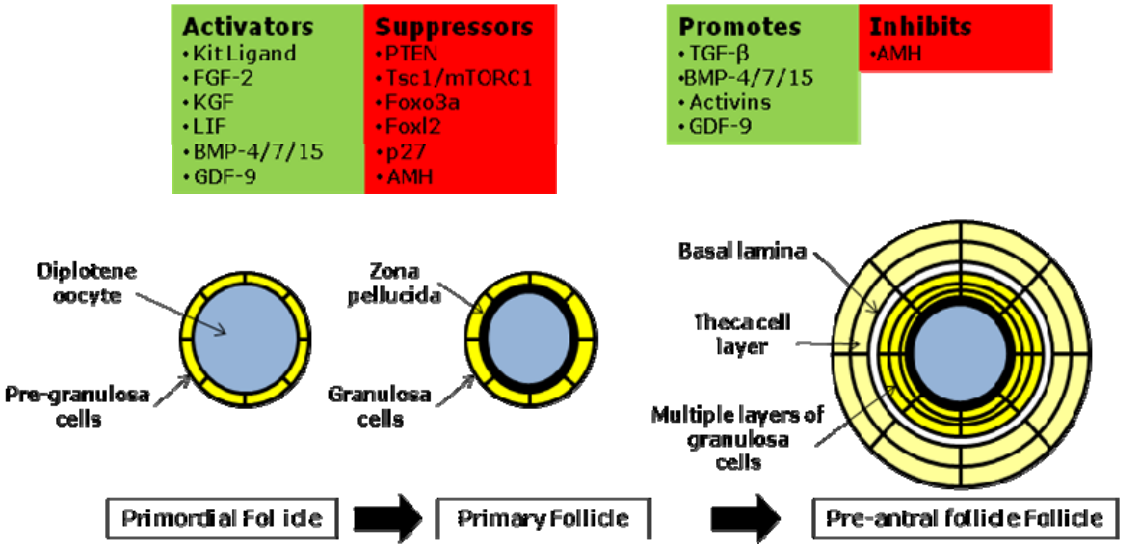

Figure 1.1: An extensive array of growth and cytokine factors acting at the paracrine and autocrine level are known to play a role in folliculogenesis from primordial follicle formation to the pre-antral stage. (Adapted from Oktem and Urman 2010).

1.2.3 Gonadotrophin responsive follicles

As the gonadotrophin independent tertiary follicles develop into small antral follicles, they become responsive to gonadotrophins. FSH receptors become detectable in follicles with only two layers of granulosa cells and are constitutively expressed from the primary to antral stage of folliculogenesis (Webb and Campbell, 2007). Although follicle growth can occur in the absence of FSH (Picton *et al.*, 1990), the proliferative effects of FSH on the granulosa cells means that at high levels it can accelerate follicle growth from the tertiary stage upwards *in vivo* (Campbell *et al.*, 2004). *In vitro*, these tertiary and small antral follicles produce a measurable steroid response to FSH and LH (McNatty *et al.*, 2010).

Although FSH is not obligatory for growth, it is conceivable that optimal oocyte quality may only be obtainable when they are present (Cortvrindt *et al.*, 1997).

During the early antral stages fluid-filled pockets begin to form in the granulosa cell layers that eventually coalesce to form the antral cavity which separates the cumulus cells from the mural granulosa. Continued proliferation of the theca and granulosa cells occurs throughout this stage, in addition to vascularisation of the theca cell layer. It is from this pool of antral gonadotrophin responsive follicles that a cohort is cyclically recruited to further grow in order to select a single dominant follicle (Oktem and Urman, 2010). Studies where FSH levels are suppressed with gonadotrophin releasing hormone (GnRH) agonists, show follicles stop developing at the small antral stage in the absence of gonadotrophins (Picton *et al.*, 1990). The antral follicle stage marks the transition from a proliferative to differentiated follicle, where marked changes in gene expression occur, specifically those involved in steroidogenesis (Webb and Campbell, 2007; Webb *et al.*, 1999).

1.2.4 Gonadotrophin dependant follicles

Follicle dependence on gonadotrophins in mice has been suggested to coincide with antrum formation, with each small antral follicle, possessing a unique FSH threshold (estimated to be at least 30% over the basal FSH value), which must be exceeded for continued pre-ovulatory development and inhibition of atresia (Figure 1.2) (Craig *et al.*, 2007; Messinis *et al.*, 2010). Follicles of this size do not occur unless critical threshold concentrations of pituitary gonadotrophins are maintained (Campbell., 2009). It is in the later gonadotrophin dependent stage of folliculogenesis that the oocyte completes cytoplasmic and nuclear maturation rendering it competent to resume meiosis. Development of the antrum is concomitant with maturation of the follicle, whereby the mural granulosa and theca cells differentiate into steroidogenic cells (Campbell *et al.*, 2007). Granulosa cells surrounding the oocyte produce and release oestrogen

into the systemic circulation through the aromatic conversion of the theca cell derived substrate, androstenedione (Knight and Glister, 2001). Gonadotrophin dependent follicles each have their own micro-environment which will transduce gonadotrophic signals differently (Webb and Campbell, 2007).

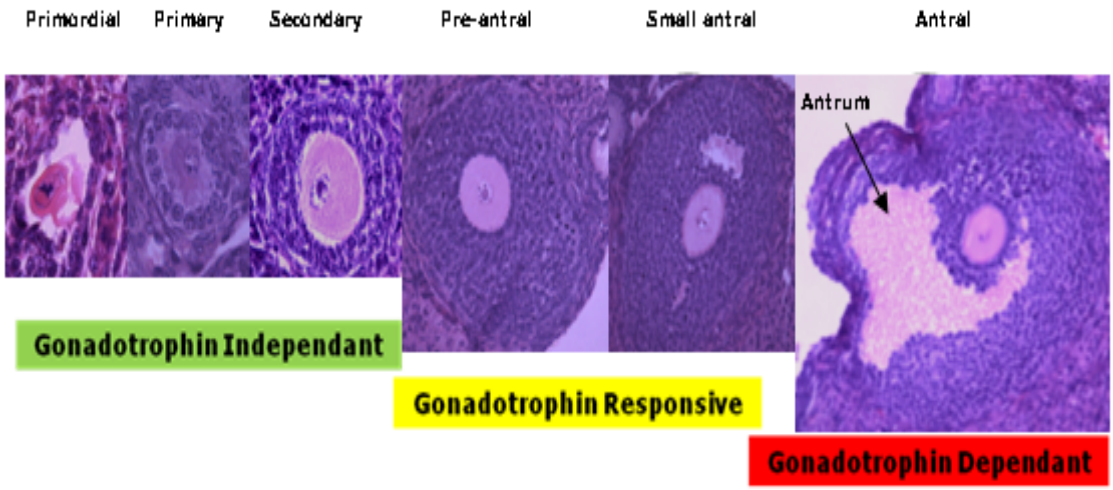

Figure 1.2: Advancement through folliculogenesis in mice is accompanied by altering dependence on gonadotrophins for further growth. During the gonadotrophin responsive stage, development can continue in the absence of FSH and LH but their presence can have an effect on development. The oocyte is arrested in meiosis I throughout follicular development and only resumes after antrum formation prior to ovulation.

1.2.5 Mouse folliculogenesis

Mouse folliculogenesis follows an analogous pathway to ovulation but takes a much shorter time period to complete than in humans, approximately 2-3 weeks from initiation of growth to ovulation (Pedersen, 1969). Differences only occur during formation of the initial PGCs. Cytokinesis during PGC proliferation is incomplete in the mouse meaning oogonia may remain inter-connected by cytoplasmic bridges forming a syncitium. It is the breakdown of these nests which leads to the formation of primordial follicles. This occurs immediately postpartum in the mouse whilst in the human it is completed in the second trimester of foetal development (Hunt and Hassold, 2008). The inappropriate breakdown of

germ cell syncitia generates polyovular follicles (contain more than one oocyte) which are common in some mouse strains with TGFβ super-family mutations. Whilst these polyovular follicles have been observed in human ovaries at birth, they are rarely found in the adult (Richards and Pangas, 2010).

Cyclic recruitment of early antral follicles is analogous in rodents with the exception that multiple follicles become dominant, proposed to be due to differing threshold levels for negative feedback between mono and poly-ovulatory species (Spearow, 1986). In hypo-gonadal and hypo-physectomised mice and rats, follicle development can progress to the early antral stage in the absence of gonadotrophins (Halpin *et al.*, 1986; Hirshfield, 1985). Prior to this stage, as with humans, mouse follicles have been shown to respond to gonadotrophins through cell divisions and differentiation, although the studies are conflicting (McGee and Hsueh, 2000).

Gene knockout (KO) studies in mice provide further support for the similarity between human and mice in the role played by gonadotrophins in regulating follicle development. FSHβ subunit KO mice were generated with no biological FSH activity. KO females were infertile, with decreased ovarian size and complete absence of antral follicles and corpora lutea (Kumar *et al.*, 1997). The morphology of primordial and pre-antral follicles appeared normal and antral follicle development could be stimulated by the pharmacological administration of PMSG and hCG (Kumar, 2009). FSH receptor KO mice also demonstrated small ovaries and a block in antral follicle formation (Dierich *et al.*, 1998), indicating that dependence on gonadotrophins becomes apparent at this stage. Complete loss of FSH function in humans displays analogous phenotypes to these mouse models and patients can respond to high doses of exogenous gonadotrophins (Beau *et al.*, 1998).

Ovarian histological analysis of infertile LHβ KO mice showed normal primary and secondary follicles but the majority of the antral follicles were abnormal containing degenerating oocytes and there were no pre-

ovulatory follicles or corpora lutea (Ma *et al.*, 2004). This could be overcome short term by administrating hCG (Ma *et al.*, 2004). Similar arrested folliculogenesis and small ovary phenotypes were observed in LH receptor KO mice (Lei *et al.*, 2001; Zhang *et al.*, 2001). This distinct LH KO phenotype compared with FSH KO mice provides genetic evidence for the distinct roles of the LH and FSH gonadotrophins during folliculogenesis (Kumar, 2009). Ovarian histology of women with LH receptor mutations show all stages of folliculogenesis except pre-ovulatory follicles and corpora lutea (Toledo *et al.*, 1996). These observations in the human and mouse support the role of FSH and LH in the final stages of folliculogenesis and the necessity of LH for the final follicular maturation and ovulation during a normal cycle (Zhang *et al.*, 2001).

1.2.6 Endocrinology of folliculogenesis

Ovarian follicle development is regulated by highly orchestrated endocrine events. Human folliculogenesis is a lengthy process taking approximately 120-180 days for a follicle to evolve from the primordial to the pre-ovulatory stage (Gougeon, 1986). The monthly cyclic selection of a cohort of follicles from the gonadotrophin responsive pool to progress past the small antral stage is governed by the hypothalamic pituitary ovarian axis. Defined as a hormone secreted from the pituitary that exerts its actions on the functioning of the gonads, the gonadotrophins are key organisers of the recurring menstrual cycle.

The hypothalamus forms the ventral part of the diencephalon in the brain. It links the central nervous and endocrine systems (i.e. hypophysis) by the controlled emission of gonadotrophins from the anterior pituitary through pulsatile secretion of the paracrine deca-peptide, gonadotrophin releasing hormone (GnRH) (Knight and Glister, 2001). FSH and LH exert their function on the ovaries, which produce steroids and gonadotrophin modulating factors, such as inhibin and activin, in response to attenuate or enhance the initial pulsatile secretion of GnRH, thereby synchronizing the oestrus cycle (Richards and Pangas, 2010). The continuation of folliculogenesis is dependent on the ability of the follicle to respond to the

gonadotrophin cues through the presence of gonadotrophin receptors. The LH and FSH receptors belong to the G-protein coupled transmembrane family and are differentially expressed throughout folliculogenesis (Jia *et al.*, 1991). FSH receptors are constitutively expressed on granulosa cells from the primary to the pre-ovulatory stage of follicular development (McNatty *et al.*, 1999). As the pre-antral follicle grows the proportion of FSH receptors exponentially increases with granulosa cell proliferation and immediately prior to antrum formation at the tertiary stage theca cells begin to express LH receptors (McNatty *et al.*, 1999; Richards, 2001). In large antral follicles granulosa cells also have LH receptors on their cell surfaces where they contribute to the production of progesterone and oestrogen (Webb *et al.*, 1999).

1.2.6.1 Follicular phase

At the luteo-follicular transition in humans, FSH levels rise due to a reduction in the negative feedback instigators oestradiol, inhibin A and the corpus luteum (CL) from the previous cycle regresses (Macklon *et al.*, 2006a). This transient increase in FSH synchronously recruits for growth a group of gonadotrophin responsive small antral follicles. The gonadotrophin responsive follicles with higher degrees of FSH sensitivity respond to the FSH increment and diverge from the pool of follicles during this cyclic recruitment (McGee and Hsueh, 2000). The continued growth and survival of this recruited follicular cohort is critically dependent on continued stimulation by gonadotrophins.

Oestrogen is thought to be produced by granulosa cells in response to FSH and LH during the gonadotrophin dependant stage of folliculogenesis by the P450 aromatase conversion of the theca cell derived substrate androstenedione (McNatty *et al.*, 1999). Through stimulation by LH, theca cells convert cholesterol into androstenedione and testosterone by cytochrome P450 side chain cleavage oxidases and 3 beta hydroxysteroid dehydrogenase respectively. FSH induced aromatase in granulosa cells subsequently converts androstenedione and testosterone into oestrogens,

such as oestradiol-17β and oestrone (Macklon *et al.*, 2006b). The actions of the gonadotrophins is proposed to be further mediated by the disulphide linked dimeric glycol-proteins inhibin and activin, which belong to the TGFβ super family (Knight and Glister, 2006). Activin *in vitro* has been shown to stimulate expression of FSH receptors (Xiao *et al.*, 1992), which through the binding of the FSH ligand may promote granulosa cell proliferation as shown in rat and human cell cultures (Li *et al.*, 1995; Miró and Hillier, 1992). Further, activin can potentially induce activation of aromatase observed in primates, rats and bovine granulosa cells (Hillier and Miró, 1993; Miró *et al.*, 1991; Shukovski *et al.*, 1993). Inhibin A, a product secreted from granulosa cells, has been shown *in vitro* to sensitise the neighbouring theca cells to the androgen production stimulatory effects of LH in human, rat and bovine (Hillier, 1991; Hsueh *et al.*, 1987; Wrathall and Knight, 1995). Inhibin A also effects FSH induced aromatase activity (Campbell *et al.*, 1996).

Collectively the hypothesised actions of activin, inhibin and other local factors and the known involvement of LH and FSH leads to an increase in serum oestrogen levels during the late follicular phase which suppresses the release of FSH from the pituitary gland (Knight and Glister, 2001). Only the dominant follicle with the greatest sensitivity to FSH is able to survive these declining levels (Baird, 1987). FSH and oestradiol are thought to induce LH receptors on granulosa cells and in combination with the constitutively expressed receptors on theca cells may enable continued development by switching gonadotrophin dependence from FSH to LH (Huirne *et al.*, 2004; Campbell *et al.*, 1999; Webb *et al.*, 1999). In addition, high circulating oestrogen levels cause intense proliferation of the endometrium, in anticipation of embryo implantation (Lessey, 2010).

1.2.6.2 Ovulation

LH is required to stimulate production of steroid hormones during various phases: follicular development, resumption of meiosis, cumulus expansion, rupture of the dominant follicle and luteinisation of theca and

granulosa cells. This generates the CL required for providing support in early pregnancy (Huirne *et al.*, 2004; Yamashita and Shimada, 2012). Persistently high levels of oestrogen produced from growing antral follicles in the follicular phase, in conjunction with elevated progesterone, concomitantly increases the quantity of GnRH produced and released whilst sensitising the pituitary to its actions, giving rise to the LH surge resulting in ovulation (Huirne *et al.*, 2004; Clarke, 1995).

In response to this peak in LH, cumulus cells surrounding the oocyte undergo expansion through the synthesis and secretion of hyaluronic acid (Yokoo *et al.*, 2008). The hyaluronan is bound to the cell surfaces by hyaluronan binding proteins (linker proteins) resulting in a sticky matrix forming in the extracellular spaces between the cumulus cells, driving the expansion (Eppig, 2001). Therefore a direct relationship exists between the level of hyaluronic acid synthesis and the extent of cumulus expansion. It is hypothesised that the transient peak in LH production is terminated by gonadotrophin surge attenuating factor (GnSAF), which antagonises the sensitising effect of oestrogen on the pituitary, thus regulating the amplitude and duration of the LH surge (Messinis, 2006). Subsequent to ovulation, there is a rapid growth of blood vessels invading the granulosa cells and the ovulated follicle is converted into the corpus luteum (termed luteinisation) and the luteal phase begins (Phan *et al.*, 2006).

1.2.6.3 Luteal phase

Following the LH surge in humans, there is an incremental increase in progesterone levels (P_4) that remain high during the luteal phase to maintain the developing CL and initial stages of pregnancy and prevent further ovulations (Stouffer, 2003). Progesterone transforms the oestrogen primed endometrium from a proliferative to a secretive tissue during the luteal phase, establishing embryo receptivity around a timed implantation window (Lessey, 2010). In mice the structure and function of the CL is only maintained for two days if mating has not occurred, or until day 10-12 of pregnancy as demonstrated in sterile mated pseudo-

pregnant mice, after which the placenta takes over (Hilliard, 1973). In rodents however, luteinisation also requires surges of prolactin secretion induced by cervical stimuli, which increases the lifespan of the CL and enables sufficient amount of P_4 to be produced to maintain pregnancy (Gunnet and Freeman, 1983). Unlike humans, mice express the enzyme 20α hydroxysteroid dehydrogenase (20α-HSD) which is involved in progesterone catabolism. Prolactin represses this enzyme and has been shown to upregulate LH receptor expression which collectively explains the increase in P_4 during luteolysis in the mouse (Albarracin *et al.*, 1994; Gibori and Richards, 1978).

1.2.7 Oogenesis and oocyte maturation

Oogenesis, is defined as the acquisition of the developing oocyte encased in the follicle to resume meiosis upon fertilisation and to progress through the pre-implantation development stages prior to zygotic gene activation (ZGA). Oogenesis occurs concomitantly with folliculogenesis (Pesty *et al.*, 2007). The acquisition of fertilising ability begins at primordial follicle activation but is only completed 1-2 days prior to ovulation. Oocytes must develop a number of competencies in order to successfully fertilise and instigate a pregnancy to term. The three aspects of oocyte maturation are: nuclear, cytoplasmic and molecular (Wassarman and Kinloch, 1992).

Nuclear maturation is the development of meiotic competence, i.e. the ability of the oocyte to overcome the meiotic block. In the mouse, it is acquired at the pre-antral to antral follicle transition as demonstrated by spontaneous resumption of meiosis when the oocyte is removed from the follicle. In humans, nuclear maturation is correlated with oocyte diameter and the ability to resume meiosis significantly increases when a diameter of 90-120μm is achieved (Mehlmann, 2005). This size is related to follicle diameter and fertilisation rates are progressively enhanced with increasing size (Trounson *et al.*, 2001). The first meiotic division is triggered at the time of ovulation and arrested in metaphase. Completion of the second meiotic division and expulsion of the second polar body occurs upon successful fertilisation and activation of the oocyte by a spermatozoon.

Resumption of meiosis is not a guarantee of full developmental competence. In addition to nuclear maturation, cytoplasmic competence is established through the complex processes involving changes in distribution, function and organisation of organelles, the most characterised being the cortical granule exocytosis that defends against polyspermy (Fulka *et al.*, 1998). Redistribution of mitochondria has also been implicated in this maturation event but many of the mechanisms have not been fully elucidated (Aerts and Bols, 2010).

As the oocyte grows it synthesises and stores large quantities of RNA, ribosomes and proteins, termed molecular maturation. At the time of the LH surge, the accumulated mRNA is rapidly translated into proteins vital for completion of fertilisation and the initial cell divisions prior to activation of the zygotic genome (Fair *et al.*, 2004). This aspect of maturation has been termed by some as "oocyte capacitation" as it reflects the intrinsic capacity of the oocyte to continue development (Fulka *et al.*, 1998). These oocyte maturation events occur cumulatively from the beginning of folliculogenesis.

1.2.8 Factors involved in folliculogenesis and oogenesis

The development of the germ cells is reliant upon bi-directional paracrine communication between the oocyte and surrounding cumulus granulosa somatic cells via transzonal projections (Matzuk *et al.*, 2002). The cumulus granulosa cells are closely associated with the oocyte in what is termed the cumulus oocyte complex (COC) (Carabatsos *et al.*, 2000). The gap junctions span from the cumulus cells through the zona pellucida to the oocyte and consist of six connexion protein subunits. These subunits join together to form the complete channel, termed the connexon on individual cell membranes, which then subsequently align to form the gap junction (Bruzzone *et al.*, 1996). These junctions permit passage of small molecular molecules such as pyruvate, amino acids, ions and signalling molecules (Wang *et al.*, 2009).

Disruption of this coupling between cumulus granulosa cells and the oocyte leads to sterility if the oocytes fail to become meiotically competent (Carabatsos *et al.*, 2000). Most of the oocyte metabolic needs required to sustain its growth and regulate its cell cycle are obtained through these gap junctions. In response, the oocyte directs the proliferation, differentiation and function of granulosa cells whilst also preventing premature luteinisation (Krysko *et al.*, 2008). The cumulus cells are vital orchestrators of oocyte maturation and development, which is why these outer cells remain in close proximity to the oocyte until the completion of fertilisation (McKenzie *et al.*, 2004). The oocyte also plays an active role in its development by secreting paracrine factors that influence gene expression and protein synthesis in granulosa and cumulus cells, whilst also maintaining an optimum microenvironment for the acquisition of developmental competence (Devjak *et al.*, 2012).

Although the importance of bi-directional communication in the acquisition of developmentally competent oocytes has been proven in rodents, it has yet to be confirmed in the human. There are several observations in the literature that suggest a role for paracrine factors in human fertility. For example, diminished levels of growth differentiation factor 9 (GDF-9) mRNA has been associated with polycystic ovarian disease, indicating a potential role in folliculogenesis (Teixeira Filho *et al.*, 2002). Mutations in both GDF-9 and bone morphogenetic protein 15 (BMP-15) have been shown to be indicators of premature ovarian failure (Laissue *et al.*, 2006). Furthermore, there is evidence to suggest the level of connexion 43 (CX43) expression (gap junction connexin encoding gene) and the strength of gap junction apposition between cumulus granulosa cells is positively correlated with IVF success in terms of pregnancy rate (Wang *et al.*, 2009). In spite of a lack of direct evidence, it can be assumed paracrine factors play parallel roles in humans as their homologous counterparts do in the mouse.

1.2.9 Angiogenesis in folliculogenesis and oogenesis

Angiogenesis is the formation of new blood vessels by intussusceptions and sprouting from pre-existing vasculature. Through a cascade of events starting with capillary proliferation, it culminates in the formation of a new microcirculation composed of arterioles, capillaries and venules (Redmer and Reynolds, 1996). Angiogenesis is an essential mechanism required for folliculogenesis and oogenesis. Angiogenesis is critical for development of ovarian follicles and has been proposed to be implemented in oocyte maturation, specifically the renin angiotensin system (RAS, discussed in section 1.5.3) which appears to be controlled by LH (Zhang *et al.*, 2010a). In addition, after ovulation angiogenesis is essential to support the developing CL (Chuderland *et al.*, 2012). The female reproductive organs, namely the uterus and ovary, undergo cyclic angiogenesis which is a pre-requisite for their optimal functionality (Reynolds *et al.*, 2002). Down regulation during COS has been shown to significantly reduce the vascularity of the ovary. However, despite this not being a factor in the number of oocytes retrieved, the effect of vascularity at the individual follicle level and subsequent embryo development and pregnancy rates was not analysed (Jayaprakasan *et al.*, 2008).

Vascularisation of follicles has been examined as a marker of oocyte and embryo quality. Early studies using doppler imaging of the ovary found that oocytes collected from poorly vascularised follicles developed into embryos of inferior quality compared to those with greater vascularisation (Nargund *et al.*, 1996). Several studies have shown that greater blood flow, specifically in the wall of the pre-ovulatory follicle may improve fertilisation rates and embryo development in IVF patients (Bhal *et al.*, 1999; Coulam *et al.*, 1999; Huey *et al.*, 1999; Du *et al.*, 2006; Shrestha *et al.*, 2006). The association between increased angiogenic indices of the follicle and retrieval rate of mature oocytes, improved embryo development and increased pregnancy rates have also been observed in mares and heifers (Silva *et al.*, 2005; Siddiqui *et al.*, 2009a; Siddiqui *et al.*, 2009b).

Several studies have shown a positive correlation between follicle vascularisation and pregnancy rate (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997; Borini *et al.*, 2005). Furthermore, there were higher incidences of aneuploidy and spindle defects in oocytes derived from poorly vascularised follicles, potentially due to the hypoxic conditions hindering oxidative metabolism and consequently lowering the intracellular pH (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997). Hypoxia leads to the formation of reactive oxygen species (ROS) which causes lipid peroxidation, inactivation of enzymes and damages both cumulus cells and the oocyte, leading to apoptosis (van Montfoort *et al.*, 2008). The concentration of ROS in follicular fluid has also been negatively correlated with embryonic development and pregnancy outcome (Das *et al.*, 2006). One study did not find this positive association between follicle vascularity and pregnancy rates after IVF; however this study did not match the infertility pathology and combined both IVF and ICSI cycles (Ng *et al.*, 2006). In addition, analysis of oocytes retrieved when no stimulation was used is lacking in the literature, so the effect of COS on the vascularity of the follicle is lacking and requires further investigation. A common response to hypoxia is angiogenesis. With our advancements in genetic understanding of cellular and molecular processes, studies are now analysing the correlation of angiogenic factors and embryo quality.

1.3 Embryo development

In mice mature metaphase II oocytes are released at ovulation in a single cumulus mass from the ovary and encounter spermatozoa in the swollen ampulla region of the oviduct (Nagy *et al.*, 2003). After 2 hours the second polar body is extruded closely followed by formation of the two pronuclei (PN) after 4-8 hours and eventually the first cleavage division 18-22 hours post insemination (Howlett and Bolton, 1985). The maternal to embryonic genomic switch occurs at the 2 cell stage in mice in contrast to humans when it occurs later at the 4-8 cell stage (Lane and Gardner, 2005; Braude *et al.*, 1988). The time period required for the embryo to develop into an expanded blastocyst is also extended in the human requiring approximately 50 hours longer in comparison to the mouse

(Taft, 2008). In parallel the time from fertilisation to implantation is also adjusted taking a mere 4 days in the mouse versus 9 days in the human (Fujimori, 2010). In spite of these species-specific differences mouse pre-implantation embryo development is morphologically similar to humans from the 1 cell zygote to the blastocyst (Figure 1.3). The subsequent three rounds of cell division occur asynchronomously resulting in spherical shaped blastomeres that are easily discernible until the 8 cell stage when they increase their contact and adhesion with neighbouring cells and form tight junctions (Fujimori, 2010). This process is mediated by uvomorulin, a calcium dependant trans-membrane adhesion molecule that belongs to the cadherin family (Nagy *et al.*, 2003).

After the fifth cell division, at the 32 cell stage, small individual fluid filled cavities form at multiple cell-cell boundaries and eventually converge to form the blastocyst cavity (blastocoel) (Kurotaki *et al.*, 2007). The regulated active transport of ions and water by the trophectoderm TEAD4-Yap system aids cavity formation and acts as a cue to activate the trophectoderm (TE) cell developmental program (Fujimori, 2010). TEAD4 is the earliest gene identified that is required for specification of the TE lineage. Ablation of the TEAD4 gene in the mouse generates a pre-implantation lethal phenotype due to failure to form a blastocoel cavity and TE, therefore preventing implantation into the endometrium (Yagi *et al.*, 2007).

The blastocyst is the first developmental stage where cell lineage is visually distinguishable with the outer TE cells contributing to extra-embryonic tissues such as the placenta and the internal inner cell mass (ICM) going on to form the embryo proper. The lineages are discernible at the molecular level also, with caudal-like transcriptional factor with homeo-domain (Cdx2) and Oct4 marking TE and ICM cells respectively. Cdx2 is required to maintain TE lineage differentiation, which provides extra-embryonic tissue (e.g. placenta) and acts as a signalling source to pattern the embryo before gastrulation (Jedrusik *et al.*, 2010). Four days after fertilisation the embryo hatches out of its protein cocoon, the zona pellucida (ZP), and directly interacts with the uterine epithelium to initiate

implantation (Fujimori, 2010). There are no preformed implantation sites in the mouse uteri but peristaltic movement evenly spaces the litters in the uterine horns (Nagy *et al.*, 2003)

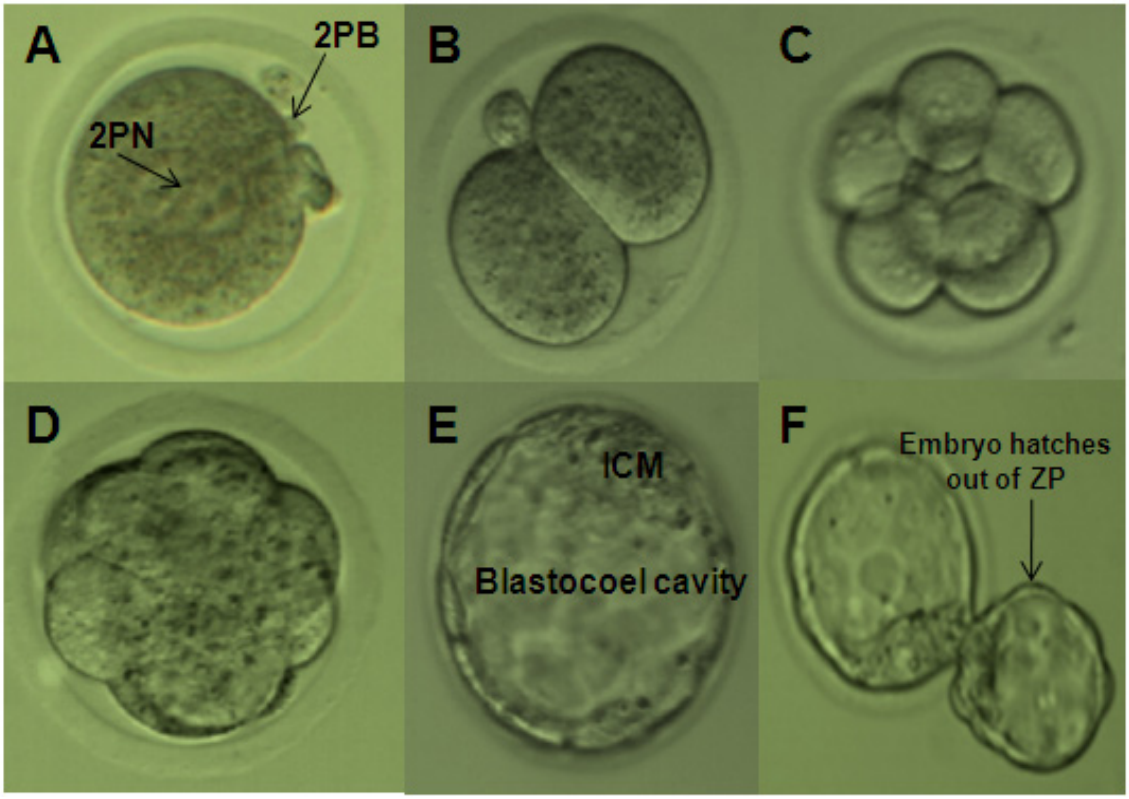

Figure 1.3: **(A)** After fertilisation the male and female pronuclei form (2PN) and the second polar body is extruded (2PB). **(B)** Following completion of syngamy the zygote divides. **(C)** Until the 8 cell stage the individual blastomeres are distinguishable. **(D)** During compaction tight junctions form and the embryo becomes a tight ball of cells called the morula. **(E)** Fluid is actively pumped into the embryo to form the blastocoel cavity that continues to expand. The blastocyst has two distinct cell types, the inner cell mass (ICM) that will form the future embryo and the trophectoderm (TE) that will develop into the placenta. **(F)** When the blastocyst is fully expanded it hatches out of the zona pellucida (ZP) in order to implant in the uterine endometrium. (Information sourced from Braude *et al* 2002).

1.4 Epigenetics in gametogenesis and embryogenesis

Epigenetics is the reversible extrinsic modification of DNA that results in altered gene expression with no change to the underlying DNA sequence

(Jurkowska and Jeltsch, 2013). This is achievable through DNA methylation, histone tail modification (acetylation/deacetylation, methylation, ubiquitination), antisense RNA and modifications in chromatin structure (Fauque *et al.*, 2007). Genomic imprinting is an epigenetic mechanism that differentially marks maternally and paternally inherited alleles at key regulatory sequences termed differentially methylated regions (DMRs), also referred to as imprinting control regions (ICRs), which mediate mono-allelic parent of origin dependant gene expression (Stouder *et al.*, 2009). Epigenetic DNA methylation patterns originate from the male and female germ lines by the action of DNA methyl transferase 3 (Dnmt3). There are various isoforms of this enzyme but Dnmt3L in collaboration with Dnmt3a, are hypothesised to be the main DNA methyl-transferases involved in maternal imprint acquisition in the oocyte as they are expressed during gametogenesis (Pradhan and Esteve, 2003).

In order to ensure appropriate sex specific imprint composition the genome undergoes widespread reprogramming during oocyte maturation and is analogous in the human and mouse (Figure 1.4). Pre-existing DNA methylation is erased during gametogenesis of PGCs with new imprints established in the germ line in an asynchronous manner during the oocyte growth phase of gametogenesis. This mechanism of erasure and reprogramming ensures abnormal epigenetic states (epi-mutations) are not transferred to the next generation. Epigenetic modifications are completed coincidently with the first meiotic division in the ovulatory follicle and are maintained throughout the rest of development (Obata and Kono, 2002). Therefore disruption in epigenetic reprogramming through the use of exogenous gonadotrophins may manifest later in gestation or after birth with life-long altercations for the individual (Khosla *et al.*, 2001).

Once established, imprints are somatically maintained through pre and post-implantation development with the aid of DNA methyl-transferase 1 (Dnmt1) (Latham, 1999). An oocyte specific transcript of this enzyme has been identified in the mouse termed Dnmt1o which is a truncated version

of the somatic isoform. This mRNA transcript is translated to protein and acts as a maternal store during embryogenesis to maintain methylation imprints until the embryonic genome is activated (Mertineit *et al.*, 1998).

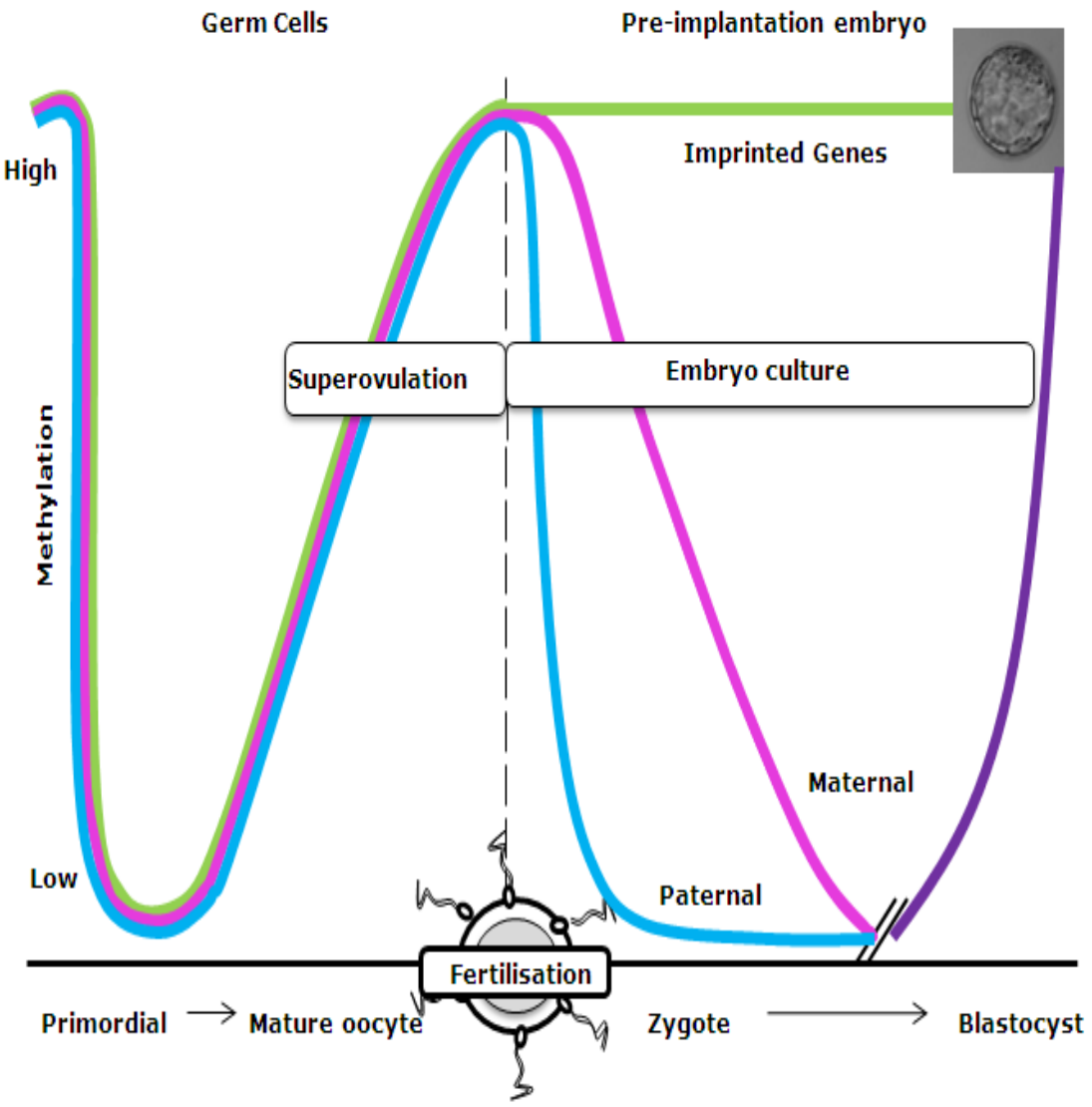

Figure 1.4: Imprinting reprogramming is achieved through the erasure/acquisition of sex specific imprints during oogenesis and is important for subsequent development. After fertilisation the paternal (blue) and maternal (pink) genomes are de-methylated by an active and passive mechanism respectively. Re-methylation (purple) begins from the 8 cell stage to the blastocyst parallel to lineage restriction. Methylated imprinted genes (green) do not de-methylate (Adapted from Reik *et al* 2001 and Dean 2003).

Whilst epigenetic imprints are sustained, global DNA de-methylation is initiated at fertilisation with paternal methylation actively removed and the maternal passively lost upon subsequent cell divisions (Figure 1.4) (Mayer *et al.*, 2000). Non-imprinted *de novo* tissue specific methylation is established by Dnmt3b upon lineage differentiation in the inner cell mass (ICM) at blastocyst formation (Reik *et al.*, 2001). During this period of dynamic epigenetic alterations, environmental factors such as ovarian stimulation and embryo culture may disrupt the ability of the pre-implantation embryo to sustain the genomic imprints initiated in the germ line, consequently having detrimental effects on future development.

Genomic imprinting is a pre-requisite for normal embryonic development as it controls early embryonic gene expression, cleavage and cell differentiation (De Rycke *et al.*, 2002). Imprinted genes play key roles in the regulation of foetal growth and placental function throughout gestation (Stouder *et al.*, 2009). Differential gene expression is hypothesised to have evolved to combat the maternal and paternal conflict of limiting and increasing foetal size respectively. The paternal alleles generally enhance placental development. In contrast maternal genes hinder placental growth therefore limiting foetal size (Jurkowska and Jeltsch, 2013).

Methylation sites are located in specific regions termed CpG Islands, in or near the promoters of genes (Figure 1.5). One function of methylation of these regions is to act as binding sites for proteins like methyl CpG binding protein (MeCP2) which recruits other proteins such as histone deacetylase that modify chromatin structure and histone protein acetylation resulting in dosage control of genes (Young and Fairburn, 2000). DNA methylation generally results in transcriptional repression of the methylated allele.

The vast majority of imprinted genes are located in clusters that are conserved between the human and murine genomes (Thompson *et al.*, 2001). Mouse studies demonstrate that disruption in epigenetic imprint maintenance results in aberrant embryo development and reduced

viability (Thompson *et al.*, 2001; Reik *et al.*, 1993). Furthermore perturbed expression of imprinted genes is associated with four broad categories of human disease: cancer, metabolic disorders, psychiatric/behavioural disorders and neuron-developmental (Paoloni-Giacobino, 2007).

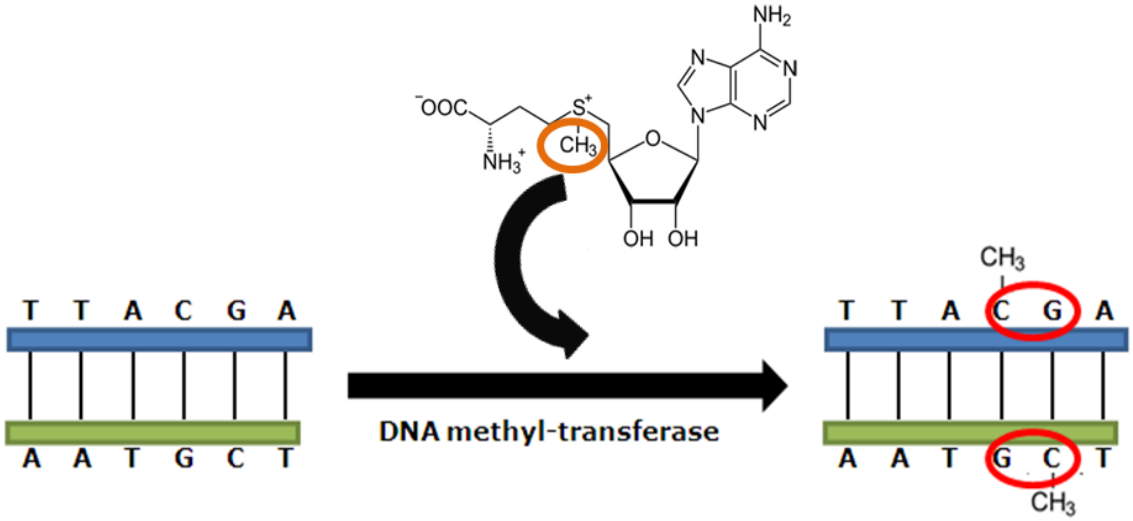

Figure 1.5: Genomic imprinting is established by methylation (transfer of the methyl group from S adenosyl L-methionine-ringed in orange, to number 5 carbon of cytosine pyrimdine ring) of CpG di-nucleotides (cytosine residues adjacent to guanidine-ringed in red) by the DNA methyl-transferase enzyme.

There is limited information available in humans on the methylation status of imprinted genes, but available data suggests imprint acquisition and maintenance during gametogenesis and embryogenesis follows a similar pattern to that reported in the mouse (Sato *et al.*, 2007). Currently, there are 96 conserved murine and human genes that exhibit parent of origin specific methylation patterns identified with more continuously being found (Wood and Oakey, 2006). The *H19* and *IGF2* gene cluster has received the most attention due to its sensitivity to *in vitro* manipulations and role in placental development (Doherty *et al.*, 2000).

1.4.1 The role of IGF2 and H19

IGF2 is a potent foetal mitogen that exerts its effects on the placenta, thus inadvertently regulating foetal growth and development (De Rycke *et al.*, 2002). It is a critical factor in placental growth and nutrient transfer.

Other attributes include oncogene and tumour suppressor gene functions. In contrast the 2.5kb *H19* transcript does not encode a functional protein, instead it is implicated in silencing the maternal *IGF2* allele so it is also crucial for foetal and placental growth (Young and Fairburn, 2000).

Both imprinted genes *IGF2* and *H19* are integral components of the Imprinted Gene Network (IGN) and are involved in embryonic and postnatal growth control (Fauque *et al.*, 2010). They are located 90kb apart from each other on the distal end of human chromosome 11p15.5 and mouse chromosome 7q11-13. Gene expression is controlled by a shared DMR upstream of the maternally expressed *H19* gene (Leighton *et al.*, 1995). Methylation is solely on the paternal allele, which acts as a chromatin boundary to the un-methylated maternally expressed *H19* gene, in addition to shielding the maternal *IGF2* gene from essential enhancer sequences and transcriptional activators such as CGBP, leading to repression (Figure 1.7) (Tilghman, 1999; Voo *et al.*, 2000).

IGF2 receptor (*IGF2r*) is a transmembrane receptor that transports *IGF2* to the lysosomes. During development, the receptor reduces the amount of *IGF2*, therefore limiting embryonic growth (Wutz *et al.*, 2001). The expression of *IGF2r* is regulated by differential methylation at two separate regions, DMR1 and DMR2. The hyper-methylated regions on the paternal allele are expressed (Sasaki *et al.*, 1992). DMR2 methylation is essential for *IGF2r* expression (Feil *et al.*, 1994). The maternally expressed *IGF2r* is also epigenetically regulated through expression of the anti-sense RNA transcript *Air*, which regulates its own allele-specific gene expression. Anti-sense RNA is defined as messenger RNA (mRNA) transcribed from an imprinted allele in the opposite direction to normal transcription leading to blockage of further transcription from this allele (Young and Fairburn, 2000).

The maternally expressed *IGF2r* is not translated into a protein but is involved in removal of the paternally expressed *IGF2* ligand by targeting it for degradation in the lysosomes. Deletion of the maternal *IGF2r* allele in

mice results in a 30% increase in birth weight, organ overgrowth and polyhydraminios, whilst no phenotypic effect was observed upon deletion of the paternal counterpart (Eggenschwiler *et al.*, 1997).

Gene expression of *IGF2* and *H19* is hypersensitive to disruptions during gametogenesis and stresses during *in vitro* culture, which is why they are used as a quasi-sensor for epigenetic perturbations (Nan *et al.*, 1998). The maternal un-methylated DMR upstream of *H19* contains four DNase-1 hypersensitive sites that are coated by nuclear proteins such as the zinc finger protein binding site CTCF to protect the region against methylation development (Figure 1.6) (Feil and Khosla, 1999). Disturbances in establishment of imprints as a consequence of superovulation or after *in vitro* manipulation can result in these sites becoming methylated, leading to a complete absence of *H19* expression and bi-allelic expression of *IGF2* (Thompson *et al.*, 2002). The *H19* CTCF1-2 region is more susceptible to disruption during ART (Fauque *et al.*, 2007).

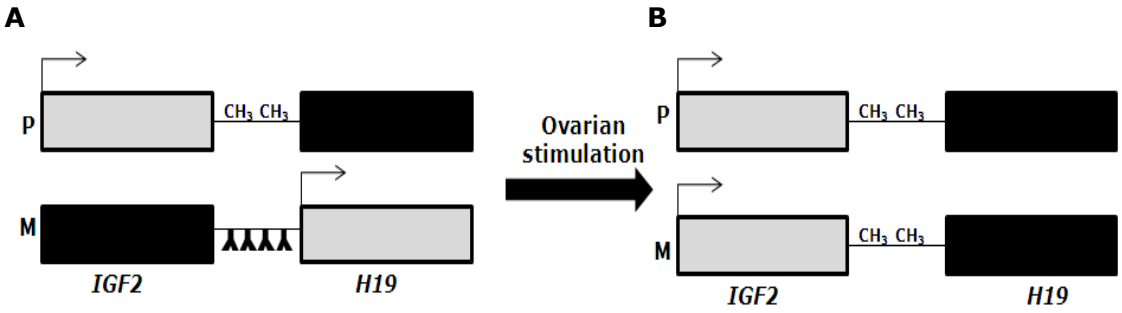

Figure 1.6: **(A)** *IGFII* and *H19* genes are regulated by methylation of the paternal DMR upstream of *H19.* **(B)** The maternal DMR upstream of *H19* has nuclease hypersensitive sites that following ovarian stimulation may become methylated (Adapted from Thompson *et al* 2001).

1.5 Implantation

1.5.1 Implantation in human

The implantation site in the human is usually at the uterine fundus (Chen *et al.*, 2012). Implantation involves the reciprocal interaction between the trophectoderm of the blastocyst and the uterine endometrium resulting in

apposition, adhesion and subsequent trophoblast invasion (Aplin, 2006). The development of new blood vessels (angiogenesis) and immunological protection from the maternal defence mechanisms is also essential (Khamsi *et al.*, 1998). This event is orchestrated by genetic and cellular signals but despite its importance in initiating a conception, very little information is available about this process. This is partly due to the inaccessibility of the uterus during the implantation event and lack of suitable models to study its mechanism of function.

Embryo implantation starts with the rapid proliferation and differentiation of uterine stromal fibroblasts, in a progesterone dependent manner, into large epithelioid-like decidual cells to form a new structure, the decidua. Decidua formation is associated with an increase in maternal angiogenesis (formation of new vessels from existing vasculature) regulated by vascular endothelial growth factor (VEGF) actions through specific VEGF receptors (VEGFRs) (Douglas *et al.*, 2009). VEGF is a 40-45 kDa, disulphide linked homo-dimeric glycoprotein, that functions as a potent mitogen and chemo-attractant for endothelial cells (Neufeld *et al.*, 1994). Uterine epitheloid cell proliferation is significantly increased after implantation indicating the embryo may provide a stimulus that enhances angiogenesis. The extravillous trophoblast can modulate vascularisation directly or indirectly via the angiogenic factor VEGF-A (Plaisier *et al.*, 2007). The decidual vasculature is the primary nutrient exchange apparatus for the embryo until the placenta becomes fully functional (Kashiwagi *et al.*, 2007).

The human placenta is extremely invasive with trophoblast cells breaching the uterine epithelium in order to sequester a blood supply for the developing placenta from the spiral arteries. *IGF2* is a key mediator of this invasion in humans and is expressed by the invasive trophoblast cells (Irving and Lala, 1995). In addition to its well documented mitogenic actions, *IGF2* also promotes differentiation and migration whilst inhibiting apoptosis which are all essential for placenta development (Han and

Carter, 2000). *IGF2* has been demonstrated to induce expression of VEGF and coincidently VEGF is primarily located in the vicinity of proliferating endothelium in the murine reproductive tract (Shweiki *et al.*, 1993). It has therefore been suggested that VEGF forms a gradient of angiogenic activity in order to induce angiogenesis in the decidua. Disruption of *IGF2* gene expression results in a marked decrease in foetal and placental weight due to impaired trophoblast invasion and insufficient vascular remodelling, which reduces maternal blood flow to the placenta, acting as a causative factor of IUGR and pre-eclampsia (Pringle and Roberts, 2007).

1.5.2 Mouse implantation

In poly-ovular mammals such as the mouse, embryos are evenly distributed in a spatial temporal pattern in the uterine horn through peristaltic movement, termed embryo spacing (Chen *et al.*, 2012). Trophoblast invasion in the murine model differs to humans because the trophoblast giant cells (TGCs) do not invade the endometrium but rather remain in close proximity to the expanding giant cell layer (Pringle and Roberts, 2007). In early to mid gestation the maternal vessels undergo extensive angiogenesis and are remodelled to form a network of dilated vessels that serve to maximise placental blood supply (Pringle and Roberts, 2007). The secretion of *IGF2* from the TGCs may induce these vascular changes and promote early placental growth. Studies demonstrate *IGF2* and both type 1 and type 2 *IGF* receptors are co-localised in developing blood vessels, thus supporting its hypothesised role in both decidual angiogenesis and placental differentiation (Pringle and Roberts, 2007).

1.5.3 The Renin-angiotensin system

The renin-angiotensin system (RAS) also plays a pivotal role in decidualisation, implantation and placentation through the regulation of blood flow, oestradiol secretion and prostaglandin synthesis (Nielsen *et al.*, 2000). It is a complex system, which is orchestrated by various

peptides and proteins. Proteolytic cleavage of angiotensinogen by renin generates the deca-peptide angiotensin (Ang) I which is ultimately converted to the biologically active octa-peptide Ang II by the angiotensin converting enzyme (ACE), as shown in Figure 1.7. Ang II has two main types of cell membrane located receptors, AT_1 and AT_2 receptors, which typically mediate antagonising actions (Matsusaka and Ichikawa, 1997). Expression of renin and angiotensinogen mRNA in the human decidua and placenta and high activities of ACE in the uterus, placenta and foetal tissues support the presence of a functional local RAS in the placenta (Nielsen *et al.*, 2000).

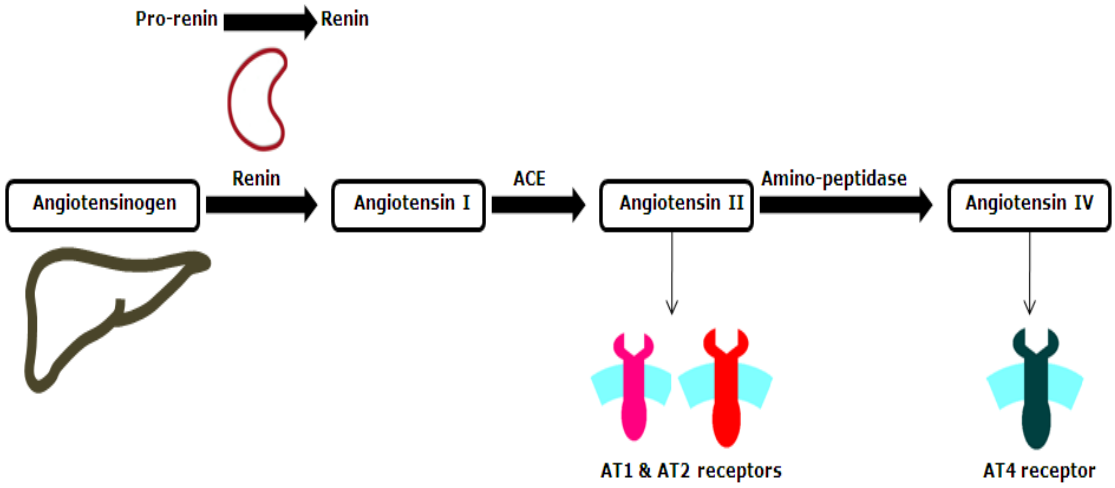

Figure 1.7: Components of the RAS. The majority of angiotensinogen is synthesised in the liver and pro-renin is converted to the biologically active rennin in the kidney.

AT_1 and AT_2 receptors are located in placenta and foetal membranes, albeit at lower concentrations in the latter (Kalenga *et al.*, 1996). Predominantly it is the G-protein coupled AT_1 receptors that are localised in the extra-villous trophoblast (EVT), cytotrophoblast, syncytiotrophoblast and in and surrounding the blood vessels of the placental villi (Li *et al.*, 1998). The positive correlation between Ang II concentration and AT_1 receptor numbers suggests Ang II may directly regulate expression of this receptor in the placenta (Kalenga *et al.*, 1996). In contrast it is the AT_2 receptors that are the predominant Ang II receptor type in the endometrium.

AT_2 receptors are down-regulated during pregnancy, most likely due to sex steroids, but the expression of AT_1 receptors remains unaltered (Matsumoto *et al.*, 1996). This can be explained by the functions of the two receptor types following binding with the ligand Ang II. AT_1 receptor stimulation causes vasoconstriction and promotes growth processes that are required throughout pregnancy. AT_2 receptors, on the other hand, inhibit cell proliferation, apoptosis and potentially vaso-dilation and mediate cell differentiation (Nielsen *et al.*, 2000). These findings led to development of the concept that changes in the relative expression of AT_1 and AT_2 receptors will determine whether Ang II promotes growth, apoptosis or differentiation, but it has yet to be assessed thus far.

Studies indicate the major physiological role of the RAS in the placenta is to interact with other systems in order to regulate tissue function. In the rat, the RAS is suggested to be involved in the differentiation of stromal cells into decidual cells during decidualisation, a pre-requisite for implantation of the blastocyst (Squires and Kennedy, 1992). Ang II, during implantation and placentation may stimulate angiogenesis, increase vascular permeability and regulate growth processes (Nielsen *et al.*, 2000). Ang II acts as a vasoconstrictor whilst also promoting vasodilatation, by stimulating synthesis of vasodilatating prostaglandins and vasorelaxants including nitric oxide (Li *et al.*, 1998). These actions will aid regulation of vascular resistance and blood flow in the placenta.

Disruptions in the RAS have been implicated in cases of pre-eclampsia and IUGR, specifically down regulation of AT_1 receptors in the placenta (Li *et al.*, 1998). Ang II has recently been suggested to play a role in early EVTs differentiation and entry into the decidua, via actions of the AT_1 receptor (Tower *et al.*, 2010). Inadequate growth and development of the villous tree leads to a reduction in gaseous and nutrient transfer capacity of the placenta which can lead to IUGR. Shallow invasion of the EVTs and impaired remodelling of the endometrial spiral arteries predisposes to pre-eclampsia (Tower *et al.*, 2010). Furthermore, decreased placental blood

flow and poor placental function may be caused by the decreased effect of Ang II in promoting secretion of vasorelaxants, ultimately leading to vasoconstriction (Nielsen *et al.*, 2000). In normal pregnancies, vaso-dilation is mediated by prostaglandin synthesis, decreasing the maternal arterial pressure.

1.6 OVARIAN STIMULATION

1.6.1 Clinical applications in ART

COS regimes are tailored to individual patients based on their pre-treatment assessment including but not limited to, ovarian reserve inferred through AMH levels or antral follicle counts, age, basal FSH levels, past response to exogenous gonadotrophins as well as the current cycle response, which is monitored through ultrasound and serum blood analyses (Jayaprakasan *et al.*, 2008). It is patient variability which makes analyses of the effects of ovarian stimulation in humans problematic. Despite the variations in treatment doses given to patients, superovulation is composed of two distinct elements, the induction of a cohort of follicles to develop to the pre-ovulatory stage, followed by the final maturation and rupture of the follicle.

FSH is used to induce multiple follicular growth (Huirne *et al.*, 2004). As previously discussed (Section 1.2.6.1), FSH levels rise at the luteo-follicular transition to recruit a cohort of follicles which develop to the tertiary stage. It has been suggested that elevation of FSH concentration above basal readings of 10-30% would be sufficient to stimulate the cyclic recruitment of follicles, termed the FSH threshold (Brown, 1978). When circulating levels decline in the late follicular phase due to negative feedback on the hypothalamus, only one dominant follicle has the sensitivity to continue growing whilst the rest of the growing cohort perishes due to atresia. The duration of elevated FSH levels termed the FSH window or FSH gate hypothesis as it was originally known was proposed as an addition to the FSH threshold theory (Figure 1.8) (Baird, 1987; Fauser and Van Heusden, 1997). Administration of FSH recruits a

variable proportion of gonadotrophin responsive follicles to persist to the ovulatory stage (Webb and Campbell, 2007).

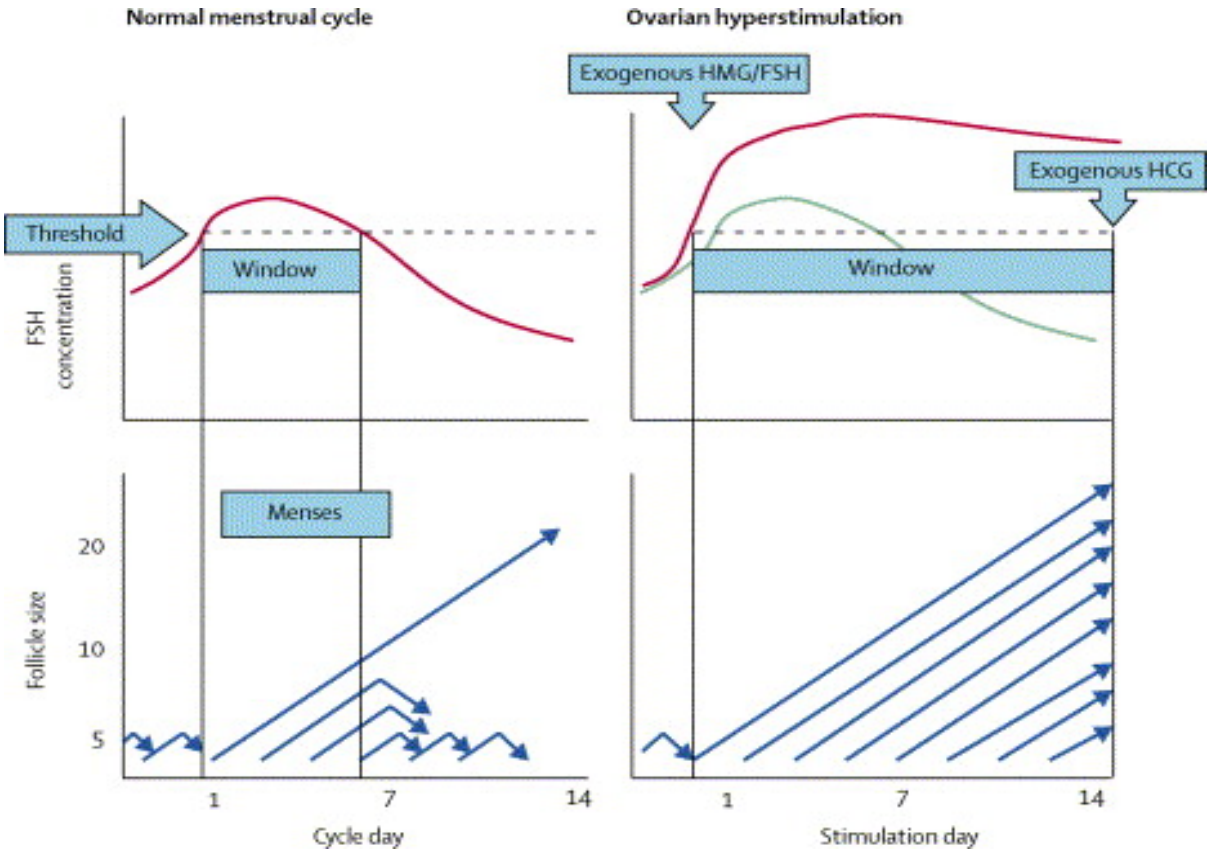

Figure 1.8: The administration of gonadotrophins extends the window when serum levels are above the threshold resulting in more follicles being recruited (Taken from (Fauser *et al.*, 2005)).

There is also an LH window hypothesis, based on the observation that low doses of LH enhances granulosa cell steroidogenesis *in vitro*, whereas high doses resulted in increased synthesis of progesterone, inhibition of aromatase activity and suppression of cell growth (Overes *et al.*, 1992; Yong *et al.*, 1992). Premature luteinisation due to high concentrations of LH may therefore occur, resulting in compromised oocyte development (Chappel and Howles, 1991). In addition, LH has been shown to modulate both oestradiol and inhibin A secretion from follicles, which could also via negative feedback, lower pituitary FSH levels, which would affect the fate of FSH dependant follicles (Webb and Campbell, 2007).

1.7 Gonadotrophins

1.7.1 Gonadotrophin structure

FSH is a hetero-dimeric glyco-protein belonging to the gonadotrophin/thyrotropin hormone family, constituting of two non-covalently linked subunits, a common α subunit and a hormone specific β subunit, as shown in Figure 1.9 (Pierce and Parsons, 1981). All β subunits possess 12 cysteines at conserved locations which form 6 intra-chain disulphide bonds that aid final conformational stability of the protein (Huth *et al.*, 1993). FSH is administered to sustain peripheral gonadotrophin levels above the threshold. It has a half-life of approximately 2 hours meaning repeated injections are required to sustain FSH levels sufficiently high enough to maintain follicular growth.

Follicle development is monitored throughout COS by the aid of ultrasound and serum hormone concentration analyses. Upon reaching the desired follicular diameter designated by the clinic (commonly 18-20mm as an indirect determiner of oocyte maturity), LH or its surrogate human chorionic gonadotrophin (hCG) is administered as a trigger to conclude maturation of the follicle, 36 hours prior to egg collection (Figure 1.9). Both LH and hCG are hetero-dymeric glyco-proteins with molecular weights of 30 and 40 kDa respectively (Hill *et al.*, 2012). Both are composed of the common α subunit found in all gonadotrophins and a hormone specific β subunit.

Despite LH and hCG possessing identical α subunits it is common practice to deliver hCG for the ovulation trigger as the modified extension of the β subunit increases the preparations half life (Huirne *et al.*, 2004). The long half life of 33 hours for hCG is partly due to the presence of four serine O-linked oligosaccharides on the extended hydrophilic carboxyl-terminus (Matzuk *et al.*, 1990). In addition to hCG having a slower metabolic clearance, it also has a stronger LH receptor binding affinity, therefore there is a potency ration of hCG to LH of approximately 6:1 (Group, 2000).

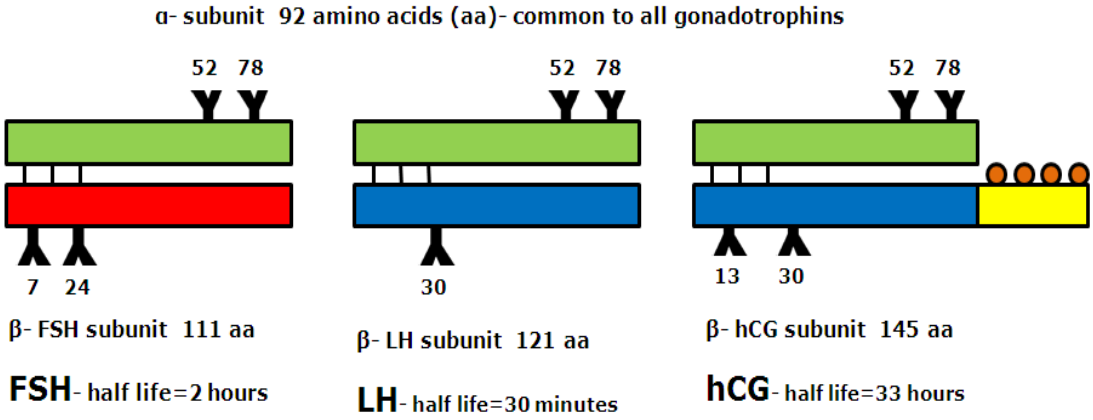

Figure 1.9: Structural representation of the hormones used in superovulation, including their respective half lives. Black branches indicate glycosylation of the protein structure, circles indicate O-linked oligosaccharides (adapted from (Macklon *et al.*, 2006b)).

To prevent early ovulation in response to a premature LH surge the endogenous hypothalamic pituitary axis must be down-regulated (Janssens *et al.*, 2000). Down regulation can be induced either by GnRH agonists which bind and stimulate the same type I GnRH receptor as native GnRH provoking down-regulation through receptor clustering and internalisation, or antagonists that cause an immediate reversible suppression (approximately 6 hours) of gonadotrophin secretion from the pituitary by competitive inhibition of the GnRH receptor (Conn and Crowley, 1994). Immature mice were used in our study to overcome the need to induce down regulation.

1.7.2 Purified gonadotrophins

Over the past three decades pharmaceutical companies have been developing more potent and purer drugs with the aim of getting maximal number of oocytes developing for ART therapies (Figure 1.10). Gonadotrophin hormones were first discovered by Ascheim and Zondek in 1927 when they isolated human chorionic gonadotrophin (hCG) from the blood and urine of pregnant women. hCG was extracted from human placenta and released on the market in 1931, under the trade name

Pregnyl (Lunenfeld, 2004). Purified urinary preparations of hCG were later released in 1940 and have survived until the present day (Huirne *et al.*, 2004). The international units (IU) of hCG was established in 1939 and was defined as the activity contained in 0.1mg of the standard preparation as judged by vaginal opening and induction of oestrus in immature rats. This ranged from 6000-8500 IU/mg (Lunenfeld, 2004).

Equine chorionic gonadotrophin (eCG) or pregnant mare serum gonadotrophin (PMSG) as it later came to be known is secreted from endometrial cups in pregnant mares and was discovered by Cole and Hart in 1930. In 1938 an international unit of PMSG was defined as 0.25mg of the standard preparation, which was defined as the total dose required to increase rat ovarian weight fivefold (Lunenfeld, 2004). Commercial preparations were released on the market soon after. It is a 60kDA glycoprotein and although being similar to hCG, in terms of terminal extension and glycosylation of the β-subunit increasing its half life, PMSG has both FSH and LH activity, so can function as a stimulator and ovulation inducer in most species by stimulating oestrogen production which then triggers the LH surge (Chopineau *et al.*, 1997; Lunenfeld, 2004). PMSG is excreted in a highly viscous gel from the endometrial cups at the point of attachment of the foetus on the surface of the endometrium from i.e. 40-130 days gestation, peaking at 50-70days. PMSG hormone is released into the peripheral circulation and prepared by collecting blood from pregnant mares near the 65th day of gestation.

As early as 1938 David and Koff described the ability of intravenously administered PMSG to induce ovulation in humans. However although it was shown PMSG produced an ovarian response during the follicular phase, attempts to induce ovulation afterwards was inconsistent and progesterone changes in the endometrium did not occur, preventing any pregnancies (Lunenfeld, 2004). Treatment of women with PMSG was also hampered due to the inter species differences resulting in formation of antibodies to the gonadotrophins, which in some cases cross reacted with their endogenous FSH and LH (Macklon *et al.*, 2006a). Due to reduced efficacy and safety of gonadotrophic preparations from animal sources the

approval of the use of PMSG in human fertility management was revoked in 1972, but its application in rodent stimulation and other mammals is still widely utilised, as due to the long half life, only a single injection is required (Lunenfeld, 2004).

The first menotrophin, human menopausal gonadotrophin (hMG) was isolated in 1949 from crude extracts of large urine pools. 30 litres of urine were required to make enough hMG for a single treatment cycle (Macklon *et al.*, 2006b). It was first released for clinical use in Italy by Serona in 1950 and the first pregnancies from this preparation were reported in 1962.(Lunenfeld, 2004). The original Pergonal 75 hMG preparation contained a combination of FSH and LH (75 IU) bioactivity in a 1:1 ratio, with an indeterminate amount of urinary protein contamination, estimated to be around 95% due to the crude and labour intensive production techniques (Lunenfeld, 2004). The original hMG preparation units used were referenced to a known standard batch called the international reference preparation (IRP-hMG) and judged on the capacity of the compound to induce oestrus in 28 day old immature rats. hMG was retrospectively standardised to IU of FSH and LH and based on the average dose given to patients (55-110IU daily). Pergonal 75 bioactivity was measured by the standard *in vivo* rat ovarian weight gain Steehlman and Pohley bioassays.

As protein purification techniques improved, with the application of immuno-affinity chromatography, the relative amounts of the active FSH ingredient increased leading to the manufacturing of urine derived preparations (urofollitrophins) containing only urinary FSH (uFSH) (Huirne *et al.*, 2004). By using an immuno-column with monoclonal antibodies for FSH, urinary hMG is filtered through with only LH and urinary proteins removed, whilst FSH is retained in the column where it can be extracted and lyophilised to a biologically pure FSH preparation. This results in an FSH product containing <0.1IU LH activity and <5% unknown urinary contaminants (Lunenfeld, 2004). This technology led to the development of highly purified hMG in the past decade, currently used today, where the LH component removed during the purification of FSH is replaced using

10IU of hCG (75IU equivalent LH bioactivity) enabling LH activity constituents to be better controlled (Wolfenson *et al.*, 2005). Based on publications in the literature, this new highly purified preparation was potentially released on the market in 2000; however the exact year is not disclosed in any publication or the company website to our knowledge.

1.7.3 Recombinant gonadotrophins

Although improvements in the purity and specific activity of urinary gonadotrophins had been achieved, the enormous quantities of urine (20-30 litres of urine per cycle) required to supply the increasing demand was a limitation. The advent of recombinant DNA technology in the 1990's led to the production of recombinant gonadotrophins, bypassing this requirement for urine and promising unlimited availability of gonadotrophins with reduced batch to batch variability (Balen *et al.*, 1999). Recombinant DNA technology enables novel gonadotrophins to be produced with longer or shorter half lives whilst the improved purity eliminates the risk of allergic reaction. Furthermore the batch-to-batch consistency compared with urinal gonadotrophins may improve the outcome of treatment. Due to the improved purity, rFSH, in addition to being expressed in IU of bioactivity, can also be expressed as protein weight (mass in µg) in so called filled by mass preparations.

Human recombinant FSH (rFSH) was developed in 1988 by stably transfecting the α- and β-FSH subunit into Chinese hamster ovary (CHO) cells via cloning plasmids (vectors) which also contained promoters so that transcription and synthesis of functional glyco-proteins initiated in recipient cells (Lunenfeld, 2004). Gonal-F® manufactured by Serono laboratories used two separate vectors to transfer the α- and β- subunits whereas Puregon® developed by Organon used a single vector containing the coding sequences of both subunit (Howles, 1996; Olijve *et al.*, 1996). No differences in clinical performance between the two recombinant products has been proven (Lunenfeld, 2004). The first pregnancy from rFSH was reported in 1992 from COS for IVF (Devroey *et al.*, 1992). rFSH was licensed for clinical use in 1995 and is now available in the form of

follitropin-α (Gonal-F®) and follitropin-β (Puregon®). Pergoveris® was recently developed which is a composition of rFSH and rLH in a 2:1 ratio combination (150 IU of follitropin α and 75 IU of lutropinα) as a mode of providing adequate doses of both FSH and LH gonadotrophins to stimulate follicle development (Bosch, 2010). Two pharmacodynamic studies have been conducted to assess the response of concomitant exposure to FSH and LH in a fixed dose. In the mouse model they displayed a synergistic effect on follicle development *in vitro* whilst in the monkey promotion of follicle growth and oestrogen production was evident (Liu *et al.*, 2002; Karnitis *et al.*, 1994). Recombinant hCG (rhCG) and LH (rLH) have additionally been developed in the 21st century (lutropin-α (Luveris®) and choriogonadotropin-α (ovidrel®)).

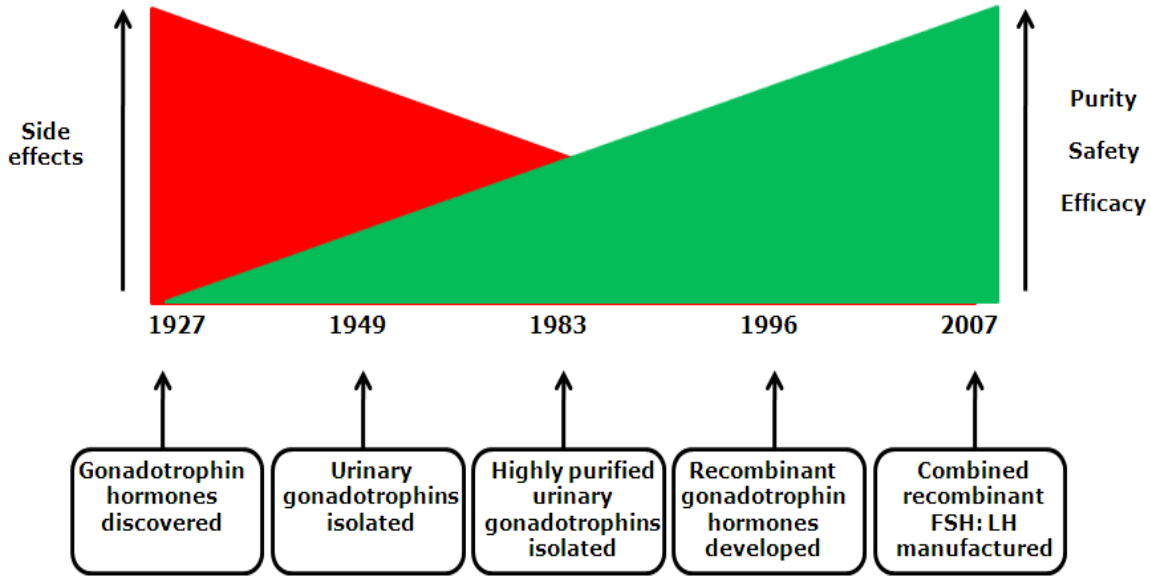

Figure 1.10: Graphic overview of the main milestones in the development of gonadotrophins for clinical application. (Adapted from Macklon *et al* 2006).

1.7.4 Efficacy of different gonadotrophins for COS

Large scale multi centre comparative trials were conducted before the clinical implementation of rFSH. Initial trials were positive with an early meta-analysis of these studies favouring rFSH over uFSH in terms of clinical pregnancy rates (Daya and Gunby, 2000). This meta-analysis however was withdrawn from the Cochrane database due to a conflict of

interest and inclusion of no up to date trials (Daya and Gunby, 2006). Many independent comparative trials have been performed since but provide variable results and are underpowered due to small sample sizes in the single centre studies. Meta-analyses and systematic reviews therefore provide the best indicators of differences in pregnancy rates and the effectiveness of the different gonadotrophin preparations.

Another meta-analysis of randomised controlled trials from the same period found no difference in cumulative ovulation rates, pregnancy rate, miscarriage rate, multiple pregnancy rate or ovarian hyper stimulation syndrome (OHSS) risk when the stimulatory effects of rFSH were compared with its traditional urinary counterpart (Bayram *et al.*, 2001). In support of this was an updated analysis which again showed no difference in pregnancy rates between hMG, uFSH and rFSH (Al-Inany *et al.*, 2003). The same research group, upon inclusion of the new highly purified hMG containing hCG as the LH substitution found clinical pregnancy rate and live birth rates in favours of hMG compared with rFSH, but only in the IVF patients (Al-Inany *et al.*, 2009). Another meta-analysis at the same time found this positive trend in favour of highly purified hMG, for both IVF and ICSI patients with a pooled increase in pregnancy rate of 4% (Coomarasamy *et al.*, 2008).

The debate as to which preparation is more efficaous was concluded to be in favour of no differences in terms of clinical success between hMG, uFSH and rFSH according to a the latest Cochrane systematic review which suggested further research in this area is unwarranted (van Wely *et al.*, 2011). However this review put all hMG studies together and did not distinguish between hMG and the new highly purified hMG which has its LH activity from hCG. A more recent review concluded highly purified hMG in long GnRH agonist cycles only, led to an increase in live birth rates of 3-4% (Hill *et al.*, 2012). It has been suggested this is due to the preparation containing both FSH and LH activity which is required for normal ovulatory follicle development. In support of this, high circulating levels of exogenous hCG in the blood of women stimulated with highly

purified hMG during mid follicular phase of the cycle has been positively associated with live birth rates (Arce and Smitz, 2013).

In normo-gonadotrophic women, supplementation of endogenous LH has been considered to be detrimental to follicle growth. The debate has resurfaced recently through increased understanding of the role of LH in optimising oocyte quality (Filicori and Cognigni, 2001). Excessive suppression of LH (<0.5 IU/L) with down regulation may impair oestrogen synthesis resulting in low oocyte yield, fertilisation and pregnancy rates with an associated increased risk of spontaneous abortion (Westergaard *et al.*, 2000; Fleming *et al.*, 2000). Therefore there may be a requirement for LH supplementation in ovarian stimulation to increase androgen production for aromatisation into oestrogen, maintaining the natural follicular milieu, potentially improving oocyte quality and thus embryo quality and implantation rates (Bosch, 2010).

As mentioned in section 1.2.8, LH exerts some of its actions on the cumulus cells surrounding the developing oocyte. These cells are involved in bi-directional communication with the oocyte which is vital to sustain oocyte viability, thus early embryo development. Therefore LH may be an essential component absent in some ovarian stimulation regimes in some patients, particularly those whose endogenous LH levels are low (Platteau *et al.*, 2004). However, as discussed in section 1.6.1, LH at high levels has been shown to have detrimental effects on oocyte development, therefore it may not be suitable for all. Current evidence suggests LH supplementation does not benefit all patients but improves pregnancy rates in poor responders and those of advanced maternal age (>35 years) (Mochtar *et al.*, 2007). In normo-gonadotrophic patients clinical pregnancy rates, implantation rates and embryo quality were comparable with traditional hormone preparations, with a slightly elevated risk of OHSS in the Pergoveris® group (Pacchiarotti *et al.*, 2010).

1.7.5 Ovarian stimulation in mice

Mice reach sexual maturity at approximately 6 weeks of age and are spontaneous polyoestrus ovulators that release 8-12 oocytes at ovulation depending on the strain (Nagy *et al.*, 2003). This value can be substantially increased with the use of exogenous gonadotrophins, reducing the number of animals required for experimentation (Nagy *et al.*, 2003; Fowler and Edwards, 1957). The established superovulation protocol for the laboratory mouse involves the (intra peritoneal) injection of PMSG to induce multiple follicle recruitment followed by hCG to induce ovulation (Nagy *et al.*, 2003; Fowler and Edwards, 1957; Luckett and Mukherjee, 1986). These injections are administered 48 hours apart in mice due to the short oestrus cycle of 3-4 days (Nagy *et al.*, 2003). Ovulation occurs 10-13 hours post hCG injection (Nagy *et al.*, 2003). The advantage of PMSG is that it only requires a single injection, however this preparation is associated with detrimental effects on the embryo and offspring, as discussed in section 1.8.2.

Since their clinical implementation, stimulation of mice with human gonadotrophins has been investigated to see whether they would yield better quality embryos as observed in humans. Although hMG increased the number of oocytes collected compared to uFSH in immature mice, the gold standard PMSG was still superior in terms of oocyte yield and fertilisation rates (Edirisinghe *et al.*, 1986). In contrast another research group proposed uFSH to be superior to PMSG based on comparable *in vitro* and *in vivo* embryo viability, potentially because this group used mature mice of an alternative strain and in addition gave repeated injections of uFSH to overcome the short half life of the gonadotrophin preparations, whereas the previous study just gave a single high dose injection which may not have been sufficient to stimulate follicle development (Muñoz *et al.*, 1995). rFSH has been shown to improve the number of mature oocytes retrieved from mice compared to uFSH which lead to improved fertilisation in the recombinant cohort, but further embryo development was not assessed (Calongos *et al.*, 2008). More recently, hMG was shown to yield comparable number of oocytes and good morphology embryos compared to PMSG but the hMG group resulted

in no live births following embryo transfer (ET) (Brooke *et al.*, 2007). This study however performed micro-injection for transgenic applications and superovulated mature mice which could have confounded the results. Comparisons of the different superovulation regimes in mice are difficult, due to the various strains and age of mice used, combined with the different doses and timing of gonadotrophin injections.

1.8 CONSEQUENCES OF SUPEROVULATION

ART involves the removal of gametes and embryos from their natural environment and subjecting them to artificial *in vitro* conditions. This environment may have profound effects on the physiology and viability of the conceptus. As development proceeds the sensitivity of the embryo to external stimuli declines as homeostatic regulation mechanisms are acquired permitting control of intra-cellular pH, metabolic controls and limiting ionic and osmotic stress (Gardner and Lane, 2005). Therefore stress inflicted on the oocyte potentially as a result of non-physiological hormone levels during COS may have a greater effect on gene function and developmental kinetics that might manifest at later stages of development (Figure 1.6). The environment the female gametes are exposed to during superovulation could impact future embryo development by multiple pathways including changes in blastomere survival (Hardy and Spanos, 2002), gene expression (Niemann and Wrenzycki, 2000) and metabolism, all of which can impede embryo development (Thompson, 1997).

1.8.1 Oocyte yield

Since the implementation of controlled ovarian stimulation (COS) in ART, the pregnancy and delivery rates have significantly improved, however the analysis of oocyte utilisation per patient portrays a conflicting story. It is estimated that the average live birth rate per oocyte inseminated in human IVF is 2-4% (Revelli *et al.*, 2011). Therefore there is significant oocyte wastage when large numbers of oocytes are produced with

stimulation. To date very few studies have analysed the outcome of the various commercial hormone preparations and protocols have on the oocyte yield. As discussed in section 1.2 ovarian follicular development is hierarchal. At any moment in time, few follicles have comparable somatic cell compositions and endocrine environments. Follicles in functionally different states will respond to the stimuli of exogenous gonadotrophins according to their developmental stage, thus the hierarchal nature of folliculogenesis may not be able to be completely overridden.

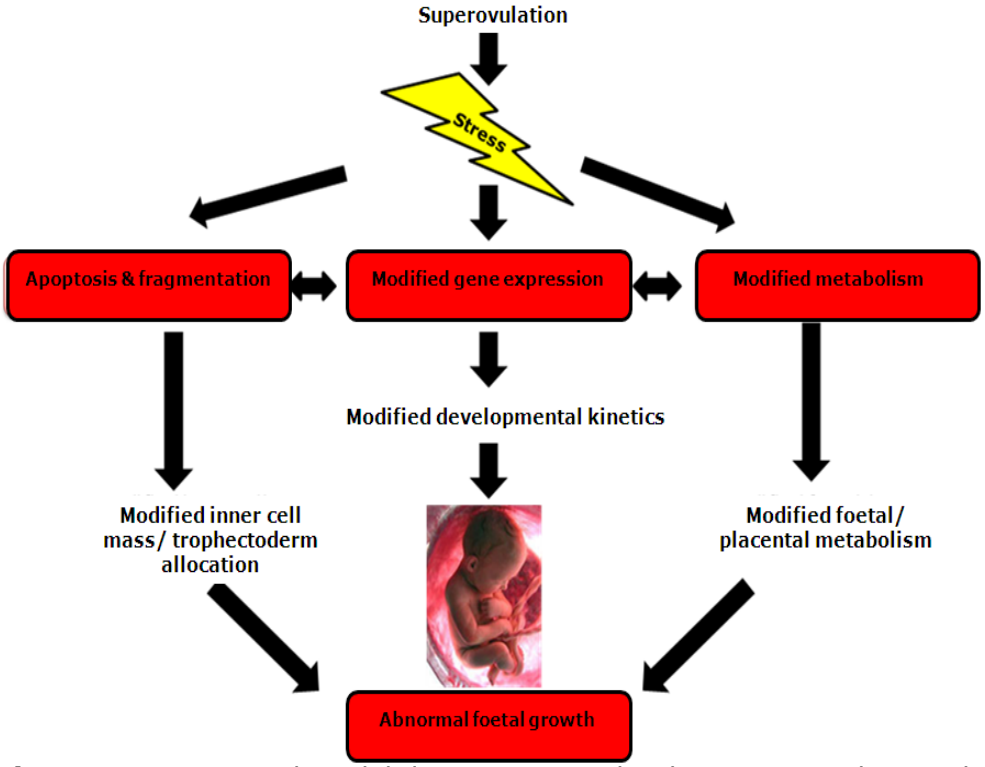

Figure 1.11: A causal model demonstrating the three main pathways that can be induced by stress on the oocyte through superovulation that manifests itself in abnormal foetal growth (Adapted from Thompson *et al* 2002).

Consequently, even though superovulation regimes increase the proportion of LH responsive follicles and thus the number ovulated, it may be that the oocytes subsequently released will have been exposed to varying endocrine and nutrient micro-environments and therefore may not all be developmentally competent (McNatty *et al.*, 2010). Oocyte quality varies widely after COS and this is most evident in high responders as it has been shown that the quantity of good quality embryos is inversely proportional to the number of oocytes retrieved (Meniru and Craft, 1997).

Oocytes recovered after superovulation may originate from immature or mildly atrietic follicles that would not have been ovulated if the current oestrus cycle was under normal physiological control (Hohmann *et al.*, 2001). In addition, the use of exogenous gonadotrophins may speed up the rate of nuclear maturation, which may prevent sufficient amounts of mRNAs to be stored, that are required for development prior to genome activation (Mehlmann, 2005). These hypotheses may explain the low success rates of ART when the number of oocytes recovered is compared to the number of offspring produced (Patrizio and Sakkas, 2009).

1.8.2 Developmental manifestations

1.8.2.1 Embryo development alterations

The use of exogenous gonadotrophins on embryo development has been particularly characterised in the mouse model stimulated with PMSG and hCG. Ovarian stimulation with this drug has been shown to reduce the proportion of Day 5 embryos and delay development to the blastocyst stage both *in vitro* (Ertzeid and Storeng, 1992; Van der Auwera and D'Hooghe, 2001) and *in vivo* (Ertzeid and Storeng, 2001). COS with PMSG was shown to increase the frequency of chromosomal abnormalities in embryos which is known to impair embryo development which may explain altered embryo development post stimulation (Chang, 1977). Total cell number of blastocysts and the number of micro villi on the cell surface was shown to be decreased following COS (Champlin *et al.*, 1987). This may potentially explain the delay observed in implantation (Van der Auwera and D'Hooghe, 2001) and the associated increased risks of neonatal mortality on mice post-implantation (Beaumont and Smith, 1975).

The detrimental effects of COS in humans are difficult to define as the majority of embryos are produced after COS and therefore have no control for comparison. The ethical limitations are also a hindrance. A small *in vitro* study has shown that elevated oestrogen, commonly observed after COS, delayed development of cleavage stage embryos and

reduced embryo adhesion rates (Valbuena *et al.*, 2001). Deleterious effects on oocyte quality and embryo aneuploidy has been shown to occur in a dose like manner in young oocyte donors (Rubio *et al.*, 2010). However, no differences were observed in a retrospective study assessing the capacity of embryos to cleave and their morphological quality grade depending on if they were obtained from a natural cycle or a COS cycle (Ziebe *et al.*, 2004).

1.8.2.2 Postnatal development alterations

The detrimental effects of ovarian stimulation with PMSG on embryo development *in vivo* and *in vitro*, implantation rates, foetal mortality and live birth weight have been well categorized in mice (Ertzeid and Storeng, 2001; Ertzeid and Storeng, 1992; Van der Auwera and D'Hooghe, 2001). Analogous ailments have also consistently been correlated in human pregnancies with singleton conceptions generated through IVF displaying more peri-natal complications than their naturally conceived counterparts, including but not limited to pre-term birth, low birth weight, intra-uterine growth restriction (IUGR) and postnatal health issues requiring hospital admission (Jackson *et al.*, 2004; Helmerhorst *et al.*, 2004; Basatemur and Sutcliffe, 2008).

In ruminants there are numerous well categorised phenotypic anomalies, which result as a consequence of alterations to imprinted genes and are collectively termed under Large Offspring Syndrome (LOS) (Smith *et al.*, 2012). Some of these reported abnormalities in foetuses and calves following the transfer of *in vitro* cultured embryos include increased rates of early embryonic death and abortion, production of large size foetuses and calves, musculoskeletal deformities, disproportionate foetal growth, abnormal organ growth, failures in development of the allantois, and abnormalities of placental vasculature and development including hydrallantois (Farin *et al.*, 2006). However, this large range of developmental abnormalities may arise through different mechanisms and is not always manifested as excessive foetal and placental growth (Farin *et al.*, 2001). The abnormal development may occur at varying degrees,

causing heterogeneity of the LOS phenotype, which makes it difficult to study the underlying cause (Farin *et al.*, 2006). The same issue is apparent in humans.

Although these developmental quandaries have not been accredited to imprint or epigenetic disturbances to date, in humans, the known involvement of imprinted genes in regulating the nutrient transfer capacity of the placenta and the phenotypic similarities displayed by animal models (LOS) post superovulation attributed to methylation perturbations, suggests errors may account for a wider spectrum of ART related complications than is currently recognized (Maher, 2005; Shi and Haaf, 2002; Khosla *et al.*, 2001). In humans, global DNA methylation in placenta and cord blood is drastically altered after IVF, with in excess of 700 genes, including imprinted genes, displaying altered methylation statuses compared to naturally conceived children, providing a potential source of the developmental and growth abnormalities (Katari *et al.*, 2009b).

Evidence indicates low birth weight is a superfluous consequence of ovarian stimulation (Pelinck *et al.*, 2010; Kalra *et al.*, 2011). Birth weight is improved, when embryos are transferred in a cryopreserved cycle. This provides some evidence that the effects of a stimulated cycle on the endometrium during a fresh cycle may also have a role in the adverse effects on neonatal outcomes (Belva *et al.*, 2008). Low birth weight has been suggested to predispose adults to stroke, type 2 diabetes and dyslipidemia, collectively referred to as metabolic syndrome as a result of low birth weight, termed the "Developmental Origins of Health and Disease (DOHaD)" hypothesis (Barker, 1995). In a Dutch cohort study consisting of IVF children aged 8 to 18 years matched to naturally conceived controls, greater systolic and diastolic blood pressure was found in the IVF children, as well as high fasting blood glucose levels (Ceelen *et al.*, 2008). This highlights the paramount importance of monitoring the long term consequences of superovulation in ART progeny.

1.8.3 Genetic perturbations

1.8.3.1 ART and imprinted disorders

In the past decade our understanding of pre-implantation development and the role genetics plays has vastly improved. In recent years focus has been directed at assessing the correlation of ART procedures with imprinting and genetic disorders. Whilst the absolute risk of developing an imprinted disorder is low, children born through ART appear to be more susceptible than those in the general population (Maher, 2005), as shown in Table 1.1. There have been numerous reports correlating genomic imprinting errors and disorders with ART, in both animal models (Market-Velker *et al.*, 2010; Menezo *et al.*, 2010; Zhang *et al.*, 2010b; Mundim *et al.*, 2009) and human subjects (Strawn *et al.*, 2010; Katari *et al.*, 2009b; Lawrence and Moley, 2008; Sato *et al.*, 2007).

The most documented imprint disorders are those that manifest through aberrant growth such as Angelman Syndrome (AS), Beckwith-Weidemann Syndrome (BWS), and Silver-Russell Syndrome (SRS), as summarised in Table 1.1 Analyses of inflicted children consistently detect methylation defects at the DMRs of *PEG1/MEST* (SRS), *KCNQ10T1* (BWS) and *SNRPN* (AS) (Fortier *et al.*, 2008). All these genes have been demonstrated to be perturbed following ovarian stimulation in a dose dependant manner (Market-Velker *et al.*, 2010). Finding the aspect of the ART procedure responsible for these risks has remained elusive. Due to the low incidence of imprinting disorders after ART it is extremely difficult to explore cause-effect relationships from clinical data. Therefore precisely defined *in vitro* models are powerful tools in defining mechanisms.

1.8.3.2 The association of methylation and COS

Imprint disorders however are merely the "tip of the iceberg" with disruption of methylation affecting the oocyte and embryonic genome on a global scale after ovarian stimulation (Shi and Haaf, 2002; Sato *et al.*, 2007). The occurrence of methylation alterations when COS was the only medical intervention to treat the infertility lends support to the hypothesis

Disorder	Phenotype	Incidence in general population	Molecular defect in general population	Estimated incidence in ART population	Molecular defect in ART cases	References
AS	Frequent laughter, severe learning handicap, lack of verbal communication, poor balance, seizures	1 in 15,000	Most cases due to deletion of 15q11.2-15q13 or paternal uniparental disomy of chromosome 15; 3-4% have hypomethylation of maternal SNRPN allele	Estimation of risk has yet to be calculated with 5 documented cases following ART	25% cases due to loss of methylation at maternal DMR 15q11-13 for the SNRPN allele	Lawrence and Moley 2008, Fortier *et al* 2008, Ludwig *et al* 2005
BWS	Foetal and postnatal overgrowth, Macroglossia, exomphalos, visceromegaly, neonatal hypoglycaemia, predisposition to embryonal childhood tumours	1 in 13,700	40-50% have micro-deletions or loss of methylation at DMR1 and DMR2 of 11p15, 20% paternal uniparental disomy, the remaining maternally inherited CDKN1C mutations, translocation or inversion of maternal chromosome 11	Largest case controlled study estimates overall risk of BWS after ART is up to 9 times higher than general population	68% of cases due to loss of maternal methylation imprint at KCNQ10T1 locus on chromosome 11	Strawn *et al*, 2010, Maher 2005, Halliday *et al* 2004, DeBaun *et al* 2003, Weksberg *at al* 2005
SRS	Intra-uterine growth restriction, limb asymmetry, clinodactyl, dimorphic facial features, gastrointestinal complications	1 in 100,000	33-67% due to hypomethylation of paternal 11p15 allele, 10% due to maternal uniparental disomy and the remaining chromosome 11 duplication and translocations or deletions of chromosomes 1, 7, 17, X	Estimation of risk has yet to be calculated with 5 documented cases following ART	Hypermethylation of the maternal PEG1/MEST locus and hypomethylation of the paternal 11p15	Bliek *et al* 2006, Kagami *et al* 2007, Amor and Halliday 2008

Table 1.1: Summary of current information on imprinted disorders linked with ART.

that superovulation is the source of methylation defects which are subsequently maintained through pre and post-implantation development as evidenced by perturbed imprints still detectable in placenta and foetal cord blood (Fauque *et al.*, 2010; Katari *et al.*, 2009b). Epigenetic marks are gained in an asynchronomous manner throughout folliculogenesis in correlation with oocyte diameter but the majority of ICRs are methylated in the diplotene stage of meiosis in late oogenesis, coincidental with antrum formation (Lucifero *et al.*, 2002). The unphysiological hormonal milieu as a consequence of exogenous gonadotrophin administration during this phase may perturb imprints.

Several studies have highlighted the occurrence of abnormal methylation patterns in oocytes or embryos after hormonal stimulation, in mice, as well as humans. Gain in methylation at the *H19* DMR has been observed in superovulated oocytes in a human ART program (Sato *et al.*, 2007). Perturbed methylation patterns have also been reported in 2 cell mouse embryos after superovulation compared to un-stimulated controls, which was associated with embryo loss during implantation (Shi and Haaf, 2002). Disruption of *H19* expression was observed in individual blastocysts and bi-allelic expression of *H19* and parallel increases of *IGF2* were still detectable in 9.5 day post coitum (dpc) placentas in mice conceived after superovulation. This highlights that methylation perturbations may be propagated through the cell lines (Fortier *et al.*, 2008; Fauque *et al.*, 2007). Quantitative differences in DNA methylation patterns have also been found in placental and umbilical blood samples taken from children conceived naturally and those born after IVF, again demonstrating the potential of aberrant methylation to remain throughout development (Katari *et al.*, 2009a).

During folliculogenesis it is the dominant and most competent follicle that is selected for ovulation over a period of time. As a result of superovulation, oocytes can be recovered from immature or mildly atrietic follicles that would not have been ovulated if the current oestrus cycle was under endogenous endocrinological control (Hohmann *et al.*, 2001). The methylation apparatus in such rescued oocytes may not be fully

competent, resulting in incorrectly established methylation imprints which are consequently maintained in subsequent cell divisions post fertilisation (Baerwald *et al.*, 2009; Young and Fairburn, 2000). Furthermore, these follicles are stimulated to develop at an accelerated pace, which may potentially be too rapid for complete oocyte maturation and synthesis of maternal factors required for acquisition and maintenance of methylation (Li *et al.*, 2010; Baerwald *et al.*, 2009).

New research supports the idea that follicles are stimulated to develop at an accelerated pace, which may potentially be too rapid for complete synthesis of maternal factors required for acquisition and maintenance of methylation (Li *et al.*, 2010; Baerwald *et al.*, 2009), by demonstrating it is not the initial methylation mechanism that is affected by ovarian stimulation but rather the mRNA stores of maternal effect gene products (Denomme *et al.*, 2011). If these stores are not sufficiently accumulated during oocyte maturation, due to them developing too quickly or being retrieved from atrietic follicles, it may render the developing embryo unable to maintain and regulate correct imprinted gene expression present in superovulated oocytes (Hohmann *et al.*, 2001). The argument that superovulation is the root of imprint and methylation disturbances in ART is strengthened by the demonstration that their incidence is increased by ovarian stimulation in mice, in a dose dependent manner (Market-Velker *et al.*, 2010). Mouse embryos obtained after superovulation with PMSG displayed twice the frequency of methylation imprint perturbations compared with those naturally ovulated which manifested in impeded embryo development (Shi and Haaf, 2002). In later gestation this aberrant imprinting was confined to the placenta suggesting the trophectoderm (TE) cell lineage may be less robust at imprint maintenance, possibly due to the exposure of these peripheral cells to the external environment of the uterine milieu (Fortier *et al.*, 2008; Mann *et al.*, 2004).

The recent discovery that altered methylation following superovulation can trans-generationally affect the germ cells of resulting offspring and further

second generations in mice highlights the importance of uncovering the origin of the disruption, in order to improve the safety and efficacy of ART in humans and prevent propagation of genetic disorders to future generations (Stouder *et al.*, 2009). The definitive cause of methylation errors is still unclear and evidently requires further investigation with longitudinal studies of ART children. A potential resistance to reprogramming during oocyte maturation may explain propagation of epigenetic defects to future generations but the mechanisms are still elusive (Youngson and Whitelaw, 2008).

In addition to disruption of imprinted genes, aneuploidies in the developing embryos have also been investigated. Aneuploidy rates are high following COS and some clinical data suggests that the incidence of aneuploidy and chromosomal abnormalities are increased when higher dose ovarian stimulation regimes are used in ART (Polinder *et al.*, 2008) (Baart *et al.*, 2007). This dose dependant manner of induction of aneuploidy has also been demonstrated in fertile oocyte donors, where lower dose protocols significantly increased fertilisation rates and the proportion of chromosomally normal blastocysts compared to higher doses, in the same patient in separate cycles (Rubio *et al.*, 2010).

1.8.4 Disruption of implantation

It is evident from the implantation rate data from infertile women undergoing IVF that the majority of embryos fail to implant. Implantation is a complicated process involving uterine cell adhesion, invasion of the endometrium, angiogenesis and immunological shielding from the maternal defence system (Khamsi *et al.*, 1998). In mice implantation rates in superovulated females are significantly lower than unstimulated animals (Ghaemi *et al.*, 2008). Failure to implant could be a consequence of endometrial receptivity inadequacy or due to an inherent problem with the embryo such as defective genes. Although both plausible, studies distinguishing between these two theories are scarce.

Blastocysts collected after superovulation with PMSG, which we subsequently transferred to non-stimulated mice for *in vivo* pre-implantation embryo development, showed reduced numbers of micro-villi on their surfaces and were consequently associated with lower chance of implantation (Champlin *et al.*, 1987). Flushed blastocysts from superovulated mice have been shown to have smaller trophoblastic outgrowths which may affect extra-cellular proteolysis required for implantation (Ertzeid *et al.*, 1993). Therefore, poor embryo quality may be due to COS.

In humans, luteolysis is advanced after the final oocyte maturation trigger in superovulation, leading to a reduction in the luteal phase and a concomitant reduction in pregnancy rate, possibly due to asynchronicity between the embryo and endometrium (Beckers *et al.*, 2003). This can be explained by the clearance of hCG levels administered mid-cycle in combination with the continued suppression of pituitary LH secretion and progesterone levels, resulting in pre-mature regression of the CL. Whist oestrogen provides feedback for gonadotrophin synthesis it also plays a functional role in stimulating cervical mucus secretion and proliferation of the endometrial lining. Reduced oestrogen synthesis as a consequence of suppression of LH production during down-regulation may impair endometrial receptivity of the embryo leading to failed implantation (Bosch, 2010). Furthermore the uterus is a vibrant source of growth factors and cytokines in whose secretion, steroids may play a role (Ertzeid *et al.*, 1993). Oestrogen has recently been shown to stimulate the angiogenic factors, vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) expression in the ovary (Ha *et al.*, 2010). Disruption due to exogenous gonadotrophin administration may cause imbalances in the uterus inhibiting implantation.

High dosages of exogenous hormones are associated with a decline in pregnancy rates (Stadtmauer *et al.*, 1994). Thus increasing hormone doses in an effort to increase the number of oocytes obtained may have detrimental consequences for embryo development. The non-physiological hormonal milieu of the uterus during COS, is detrimental to endometrial

receptivity, with the elevation of oestrogen dictating the duration of the implantation window (Kolibianakis *et al.*, 2002). Luteal phase progesterone supplementation to compensate these effects of down-regulation on the females' ability to sustain a pregnancy is now common practice in COS (Macklon *et al.*, 2006a).

1.9 HYPOTHESIS, AIMS AND OBJECTIVES OF RESEARCH

Despite the poor implantation rates after ART and the increased prevalence of disorders in resulting children, there is paucity of information on the definitive identification of the causal factor. The one thing the majority of ART treatments have in common is superovulation, therefore with our enhanced appreciation of the importance of genomic profiles, particularly imprinted genes, it is pertinent to assess the protocols to identify what is responsible for ART consequences. The overall objective of this research therefore was to assess the impact of different gonadotrophin preparations on oocyte yield, oocyte quality and subsequent embryonic and foetal development.

Various commercial preparations of gonadotrophin hormones have been developed and are in clinical use today. Adequate investigations into the effects of these preparations in superovulation protocols are lacking. It is the specific aim of this research to examine 3 different human gonadotrophins, hMG, rFSH and combined rFSH and rLH in a 2:1 combination (Pergoveris) in mouse superovulation on the outcomes of oocyte yield, embryo development, number of pregnancies, health of offspring and ovarian pathology. The results of this will be compared to the un-stimulated negative control and PMSG the COS positive control. This will be achieved by completing the following specific objectives:

1. Assess the impact of the treatments on oocyte quality. Cumulus cell morphology will be categorised and after removal will be analysed for the angiogenic factors *VEGFA, PEDF* and *MYHII*. The maturity status of the oocytes will then be assessed and fertilised zygotes

will remain in culture. The hypothesis is that ovarian stimulation will reduce the quality of oocytes.

2. Assess the impact of the treatments on embryo quality. Embryos will be cultured for 5 days with daily monitoring. Upon reaching the blastocyst stage, embryos will be graded and stained to provide further information on quality in terms of cell numbers and apoptosis rates. The hypothesis is that stimulation with PMSG, will be more detrimental than human gonadotrophins.
3. Assess the impact of the treatments on gene expression. Specifically we will look at the expression of imprinted genes *H19, IGF2* and *IGF2r* and the RAS genes *ATR1, ATR2* and *VEGFA* in blastocysts. The hypothesis is that blastocysts following treatment with PMSG will have greater differences in gene expression compared to naturally ovulated control, than those stimulated with human gonadotrophins.
4. Assess the impact of the treatment on resulting offspring following embryo transfer. After *in vitro* culture, blastocysts will be transferred to pseudo-pregnant females and the pups born will be analysed for total body weight and relative organ weights for the 3 weeks prior to weaning. The hypothesis is that pups born following treatment with human gonadotrophins will be more comparable to non-treated controls, than those born after PMSG stimulation.

CHAPTER 2: MATERIALS AND METHODS

The following chapter describes those methods common to all the *in vitro* trials carried out for this study. Any changes made or additional techniques performed for a specific study are detailed in the relevant chapter. In order to assess the consequences of superovulation on embryo and subsequent development, mouse zygotes were obtained from females superovulated with different protocols and cultured *in vitro.* Pregnant mare serum gonadotrophin (PMSG), human menopausal gonadotrophin (hMG), recombinant follicle stimulating hormone (rFSH) and rFSH and recombinant luteinising hormone (rLH) in a 2:1 ratio were used in the superovulation sub-experiments, allowing comparisons to be made between the different regimes in terms of embryo yield and quality. Zygotes obtained from naturally mated mice were used as negative controls for *in vitro* development rate and gene expression. Following collection, the culture and manipulation of embryos were kept constant regardless of the experimental group, meaning only the affect of the superovulation protocols were being analysed.

2.1 OVARIAN STIMULATION

All mice and embryos utilised in this research were ethically approved and regulated under the Scientific Procedures Act of 1986. All research conducted was covered by the Home Office under license numbers 40/2914 and 40/3480 held by Professor Bruce Campbell. Trained staff with their own personal Home Office license euthanized the mice. Four-five week old (C57BL/6 x CBA)F1 hybrid female mice (Charles River UK Ltd, Margate, UK) were ordered throughout the study. Mice were housed in groups of 4 in enclosed IVC cages at suitable conditions with food and water *ad libitum,* on a 12 hour light and 12 hour dark cycle within the Biomedical Sciences Unit (BMSU) at the University of Nottingham, for a minimum of 7 days to synchronise the oestrus cycles (Lee-Boot Effect).

Upon reaching the desired weight of approximately 17g at 5-6 weeks of age, mice were randomly allocated to the superovulation sub-experiments to minimise external influences such as genetic background of the donor mouse and culture conditions. Eight week old male B6CBAF1 mice were also maintained in the animal unit for a maximum of 6 months to ensure optimal mating capacity.

2.1.1 PMSG stimulation

Five-six week old (C57BL/6 x CBA)F1 female mice were superovulated by injection with 5IU of Pregnant mare serum gonadotrophin (PMSG) (Intervet, Buckinghamshire, UK) into the peritoneal cavity at 11am and 47 hours later, at 10am, 5IU of human chorionic gonadotrophin (hCG) (Chorulon, Intervet) was administered. Females were monogamously caged with males of the same genotype (B6CBAF1) and mated overnight.

2.1.2 hMG, rFSH and Pergoveris stimulation

Five-six week old female mice were superovulated by injection with the gonadotrophin preparations summarised in table 2.1 twice daily at 8.30am and 5.30pm for two consecutive days. On the third day at 10am, 5IU hCG was administered and females were monogamously caged with males and mated overnight.

Table 2.1: Summary of human gonadotrophins used

Preparation	Manufacturer	Concentration
hMG	Menopur, Ferring Pharmaceuticals	0.5 or 5IU
Pergoveris	Merck Serona	0.5 or 5IU
rFSH	Gonal-F, Merck Serono	0.5 or 2.5 or 5IU

2.2 Naturally mated control

The natural ovulation (negative *in vitro* control) group of 5-6 week old B6CBAF1 females were housed with males of the same genetic stain for three consecutive nights. Upon vaginal plug detection each morning following mating, females showing evidence of copulation were euthanized and embryos and ovaries obtained, whilst unplugged females remained with the males until the following morning when evidence of mating was investigated again. If on the third morning female mice had failed to plug they were not used in the experiments. The majority of female mice plugged on the third night of housing with the male, in accordance with the literature, known as the Whitten effect (Whitten, 1956).

2.3 EMBRYO COLLECTION AND CULTURE

The presence of copulation plugs indicating day 1 of pregnancy was assessed the following morning and all female mice were euthanized by a schedule 1 approved method. Cervical dislocation was the method chosen as it has been shown to be an effective technique for obtaining intact oocytes (Roustan *et al.*, 2012).

2.3.1 Embryo collection

All culture media used was purchased from Vitrolife[1] and prepared fresh on the day of usage. Reagents and consumables were purchased from Sigma Aldrich UK unless otherwise specified. All procedures were carried out on a heated stage (LINKAM CO102, Surrey, UK) with the temperature in the culture medium at 37°C. After euthanasia embryos were collected as quickly as possible to avoid the deleterious effects observed through extended handling in GMOPS[2]. Mice were sprayed with 70% alcohol (458600, Sigma Aldrich) and following removal of the dermis and dissection of the peritoneal wall with scissors, each ovary and uterine horn

[1] See Appendix 1 for media compositions

[2] See Appendix 2 for experimental data on GMOPS

was teased from the abdomen with the aid of forceps and the ovary and oviduct excised (Figure 2.1). Collected tissue was immediately placed in a centre well dish (BD Falcon, 353653, Oxford) containing 1ml of warmed G-MOPS^{TM} in the well and 3ml in the moat (Vitrolife, Warwick, UK) which had previously been supplemented with 5% Human Serum Albumin (HSA) and incubated in a non-gassed humidified chamber at 37°C (PLANER BT37GP, Middlesex, UK). Left and right ovaries and oviducts were kept separate throughout.

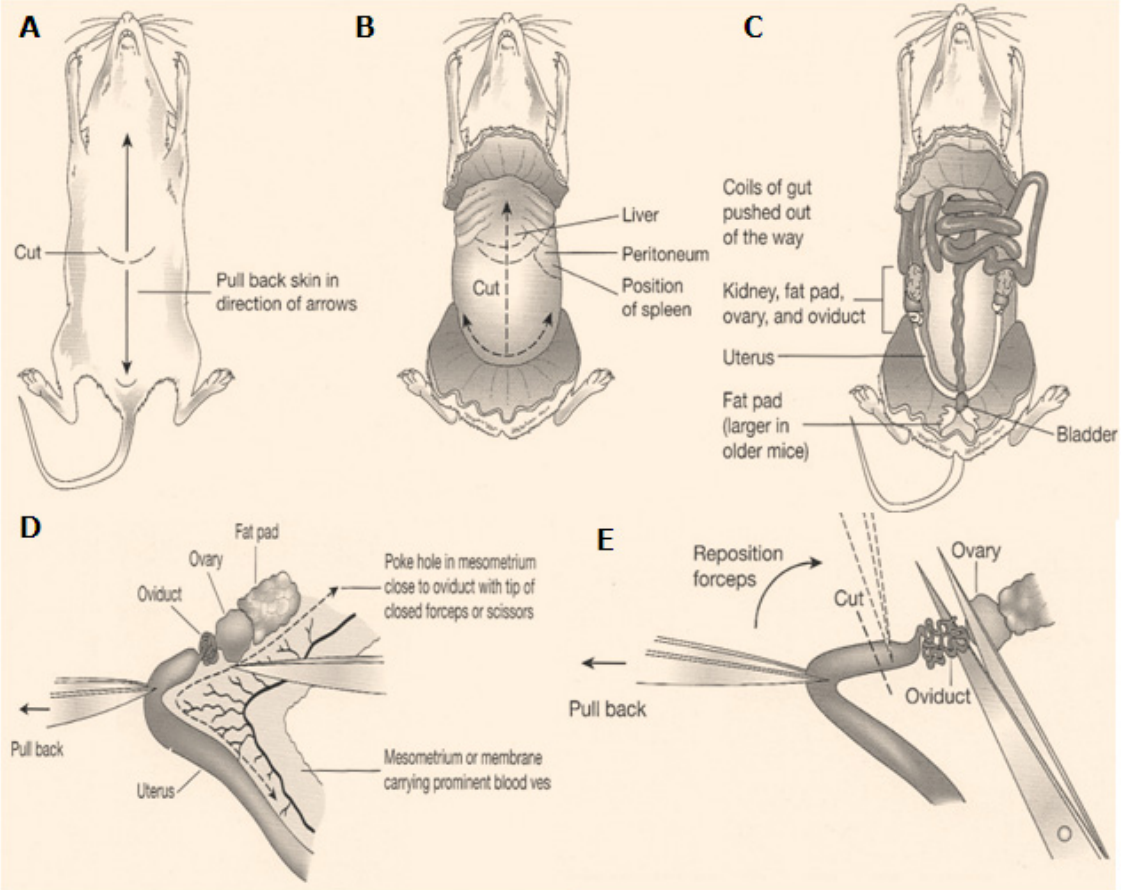

Figure 2.1: **A** Following cervical dislocation, mice were spread with 70% alcohol. **B** The skin pulled away from the abdominal region. **C** The peritoneum cut to expose the internal abdominal organs. **D** The bi-cornuate uterus, oviducts and ovaries located and oviduct extracted.

The embryo cumulus complex was removed from the oviduct by tearing the swollen ampulla region with a sterile needle (BD Microfine, 324899,

Oxford, UK) under a stereoscopic dissecting microscope (OLYMPUS SZ40, Southend-on-sea, UK) with a heated stage (Figure 2.2). One cell embryos collected were denuded in 50µl drops of 80IU bovine testes derived hyaluronidase (H4272) under 3ml of mineral oil (M8410) in 35mm cell culture dishes (Corning, 430165, Sigma-Aldrich) and washed twice in 50µl drops of GMOPS under oil before being put into culture using a 10µl Gilson pipette.

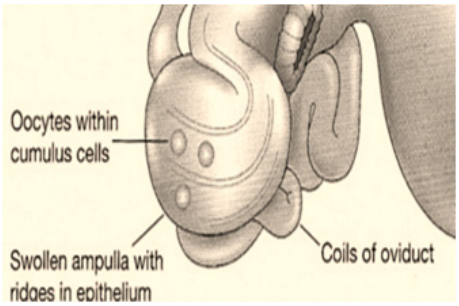

Figure 2.2: Schematic diagram depicting the oviduct (Nagy *et al.*, 2003).

2.3.2 *In vitro* embryo culture

Throughout all procedures and handling of embryos 0.130-0.133mm denudation pipettes (Swemed, 14302, Vitrolife) were used unless stated otherwise. All dishes were prepared a minimum of 1½ hours in advance to allow equilibration (Doherty and Schultz, 2000). Day 1 pronuclear embryos were transferred to 60mm culture dishes (BD Falcon, 353652) containing twenty five 5µl micro-drops of G-1 culture medium (Vitrolife) supplemented with 5% HSA overlaid by oil pre-equilibrated in a benchtop incubator at 37°C, 6% CO_2 (MINCs, COOK). After transferral embryos were incubated at 37°C in a humidified atmosphere with 6% CO_2 (Sorvall Heraeus BB6220 Incubator, Kendro Laboratory Products, Basingstoke, UK) for continued single embryo culture. When embryos reached the 4-8 cell stage assisted hatching (Refer to section 2.4.1) was performed before transferring them to 60mm culture dishes containing twenty five 5µl drops of G-2 medium (Vitrolife) supplemented with 5% HSA overlaid with oil and incubated again in a closed incubator system at 37°C and 6% CO_2 and atmospheric O_2.

2.3.2.1 Development monitoring

Assessment and documentation of cleavage was performed at a pre-fixed time daily from embryo collection until the blastocyst stage using image capture software (XYCLONE, Hamilton Thorne Biosciences, Beverly, MA). The *in vitro* development of embryos was not affected by the daily removal of the embryos from the incubator for analysis as exposure to atmospheric conditions was as brief as possible and the temperature was maintained by a heated stage.[3]

Towards the end of the study embryos were also cultured in an Embryoscope™ Incubator (Parallabs Ltd, UK/Unisens FertiliTech A/S, Denmark) which has an integrated inverted microscope and imaging system for embryo viewing. Digital images were acquired every 20 minutes in 7 focal planes, permitting time lapse analysis. Subsequent to collection and denudation embryos were loaded individually into the 25µl drops of G-1 media under 2ml mineral oil in the embryoscope slide. Twelve embryos were loaded per slide, and returned to the Embryoscope for further culture. Media change was performed by washing out the wells with pre-equilibrated G-2 media using a footprint technique. This self contained incubator uses a tri-gas mix to control the CO_2 and O_2 levels at 5% and 6% (lower than 21% atmospheric utilised by the other incubator).

2.3.2.2 Blastocyst grading

Blastocysts were classified into different developmental stages: early (blatoceal cavity not fully formed), fully expanded (Blastocoel completely filling cavity and expanding the zona pellucida), hatching and hatched. Blastocysts were further graded according to the detailed grading system developed by Gardner and Schoolcraft (Gardner and Schoolcraft, 1999) as shown in Table 2.2.

[3] See Appendix 3 for experimental data on daily monitoring of embryos.

Table 2.2: The three separate quality scores assigned in blastocyst grading. (Adapted from Gardner and Schoolcraft 1999).

Expansion Grade	Blastocyst development and stage status
1	Blastocoel cavity less than half the volume of the embryo
2	Blastocoel cavity more than half the volume of the embryo
3	Full blastocyst, cavity completely filling the embryo
4	Expanded blastocyst, cavity larger than the embryo, with thinning of the shell
5	Hatching out of the shell
6	Hatched out of the shell
ICM Grade	**Inner cell mass quality**
A	Many cells, tightly packed
B	Several cells, loosely grouped
C	Very loose cells
TE Grade	**Trophectoderm quality**
A	Many cells, forming a cohesive layer
B	Few cells, forming a loose epithelium
C	Very few large cells

2.3.3 DAPI staining of arrested embryos

Embryos without distinguishable polar bodies or pronuclei that remained uncleaved at the time of media change were classed as unfertilised and underwent oocyte staining to determine the maturity status. In addition embryos which had ceased cleavage at the 2 cell stage onwards were also stained to determine the putative cause for the developmental arrest.

Subsequent to 5 minutes wash in Dulbecco's Phosphate Buffered Saline (PBS) (D8537, Sigma) supplemented with 2% bovine serum albumin (BSA) (Albumin Bovine, A2153, Sigma) (PBS-BSA), oocytes were incubated at room temperature in 4% paraformaldehyde (PFA) (43368, Alfa Aesar, Lancashire, UK) for 20 minutes and washed again in PBS-BSA before incubation in 0.5% Triton-X100 (T-9284, Sigma) mixed in PBS-BSA for 15 minutes. After a final wash in PBS-BSA oocytes were grouped in 10s and transferred onto a slide with drops containing VECTASHIELD mounting medium with with 4',6-diamidino-2-phenylindole (DAPI) (VECTOR Laboratories, Burlingame, California, USA) and the cover slip sealed with nail varnish. Analysis of maturity status was performed using fluorescent light microscopy.

2.4 GENE EXPRESSION

2.4.1 Assisted hatching

All biopsy procedures were performed using an inverted tissue culture microscope (OLYMPUS IX70, Integra TI) with an in-built heated stage whilst the embryos were in 5µl drops of G-PGD (Vitrolife) supplemented with 5% HSA under oil in 60mm ICSI dishes (BD Falcon, 353665). At the 4-cell stage an opening in the zona pellucida was drilled with a 260-*µs* pulse from a non-contact laser (XYCLONE, Hamilton Thorne Biosciences, Beverly, MA) to promote herniation of the trophectoderm upon further development (Figure 2.3).

Figure 2.3: **A** An 8 cell stage embryo suitable for drilling **B** Laser is directed at area with most distance between zona pellucida and oocyte **C** A single pulse of the laser is sufficient to thin the zona.

2.4.2 Trophectoderm biopsy

Trophectoderm biopsy was only performed on blastocysts hatching a sufficient distance away from the (ICM) (Figure 2.4). Trophectoderm biopsy was chosen to assess mRNA profiles based on the observation that in humans, the trophectoderm mRNA profiles correlated with embryo viability (Jones *et al.*, 2008). Differential staining was performed on blastocysts hatching at the site of the ICM. Approximately 5 trophectoderm cells were aspirated with a 25μm sharp, thin walled biopsy pipette (Biomedical Instruments, Zöllnitz, Germany) and detachment achieved with repeated 300-*μs* pulses of the laser (staccato), whilst the pipette was drawn away from the embryo, which was held stationary with a 9-17μm thick walled blunt holding pipette (Swemed, 14317, Vitrolife). A video of which is shown on the CD-Rom accompanying this thesis.

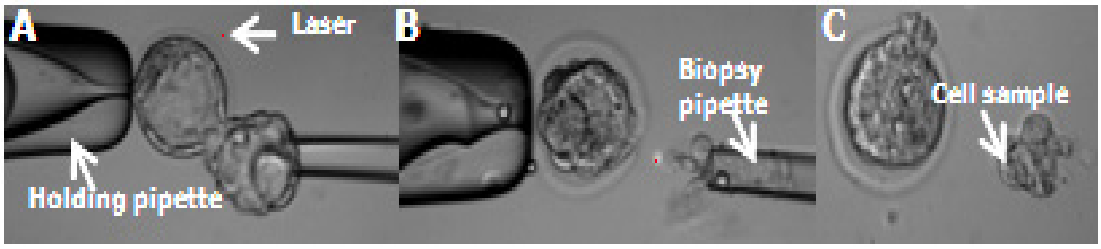

Figure 2.4: **A** Blastocysts chosen that are hatching away from the inner cell mass. **B** Trophectoderm cells are aspirated and whilst pulled away from the embryo are detached with the aid of the laser. **C** Cell sample for further analysis.

2.4.2.1 Viability 3 hours post biopsy

Biopsied cells were designated for genetic analysis and embryos returned to culture in G-2 for 3 hours to check for evidence of re-expansion indicating viability (Figure 2.5). Time lapse videos of re-expansion were obtained using the aforementioned Embryoscope, in addition to the Primo vision time lapse embryo monitor (Cryo Innovation, UK), which is an image capture device placed inside the incubator. Biopsied blastocysts were transferred to 16 well, well of the well (WOW) dishes (Cryo Innovation) containing 25µl pre-equilibrated G-2 media under mineral oil. Embryos that survived the biopsy were re-graded pooled and triple stained to assess the biopsy effect on future quality.

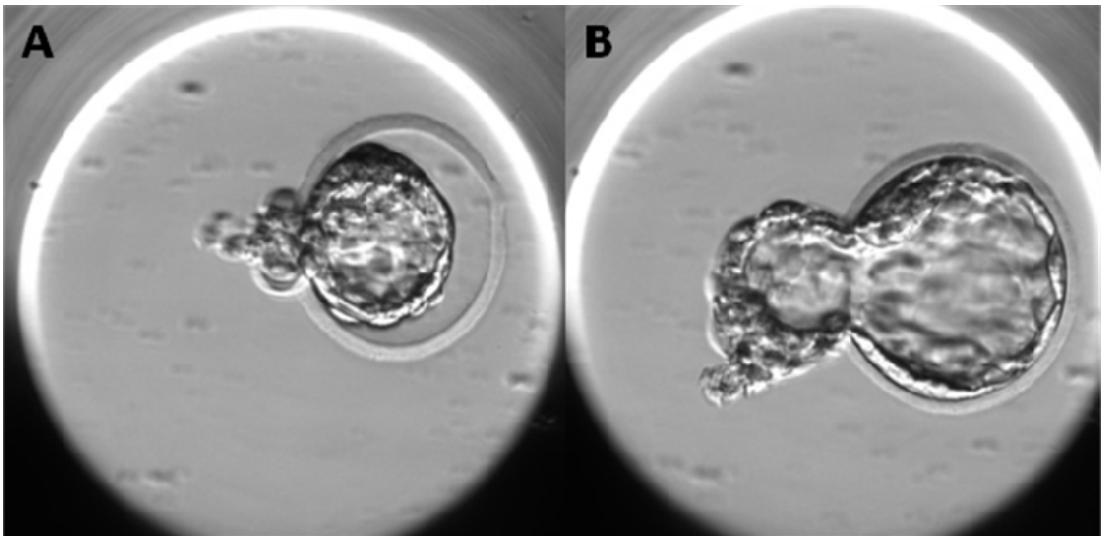

Figure 2.5: Example time lapse images from the Embryoscope **A** Biopsied blastocysts collapse due to loss of blastocoel fluid during the procedure. **B** Re-expansion due to replacement of fluid indicates survival.

2.4.2.2 Triple fluorescence staining-DAPI, PI, Tunel

Biopsied blastocysts and those blastocysts that were unsuitable for trophectoderm biopsy due to location of hatching in the vicinity of the ICM, were triple stained to determine the quality of blastocysts expressed as total cell numbers (TCN), a ratio of ICM:TE cells and to identify apoptotic cells. All staining protocols were conducted in glass embryo dishes with lids (Fisher Scientific, SDE90, Leicestershire, UK) and embryos were moved through all incubation stages using a 10µl Gilson pipette.

Cavitated blastocysts were washed once with PBS-BSA at 37°C. Trophectoderm cells (TE) were permeabilised with PBS-BSA supplemented with 0.2% Triton X-100 for 20 seconds. After being thoroughly washed in PBS-BSA, TE cells were stained red by incubation in 10µg/ml of Propidium Iodide 95% (PI) (P4170, Sigma) in PBS-BSA for 10 minutes in the dark at 37°C. Subsequent to this step all remaining incubations were performed in the dark.

After three wash steps in PBS-BSA blastocysts were fixed in 4% PFA for 15 minutes at room temperature. Following two wash steps in PBS-BSA blastocysts were further permeabilised in 0.2% Triton X-100 supplemented with 0.1% sodium citrate for 20 minutes at room temperature. Apoptosis was detected using the Click-iT® TUNEL Alexa Fluor® 488 reaction kit according to the manufacturer's instructions (C10245, Invitrogen). DNA strand breaks were induced in the positive controls through incubation in 100µl DNase I solution (composed of: 89µl deionised water, 10µl DNase I buffer and 1µl DNase I) for 30 minutes.

After blastocysts were washed twice in distilled water apoptosis was detected by incubation in 6µl droplets of Terminal deoxynucleotidyl Transferase (TdT) reaction buffer under oil for 10 minutes at room temperature. Further incubation in 100µl drops of TdT reaction cocktail (composed of: 94µl of TdT reaction buffer, 2µl of ethynyl modified nucleotide EdUTP and 4µl TdT) for 60 minutes at 37°C was performed following washing of blastocysts in PBS-BSA. Blastocysts were washed twice in PBS-BSA and incubated in 100µl drops of Click-iT reaction cocktail (composed of: 97.5µl of Click-iT reaction buffer and 2.5µl of Click-iT reaction buffer additive) for 30 minutes at room temperature. After a further wash in PBS-BSA, blastocysts were left in 100µl drops of 1mg/ml solution of Hoechst (BisBenzimide H33258, B1155, Sigma) in PBS for 15 minutes at room temperature.

After being washed once in PBS-BSA and once in a 50:50 solution of PBS-BSA with VECTASHIELD mounting medium with DAPI, blastocysts were finally mounted on a diagnostic microscope slide (Thermoscientific, ER-308B-CE24, Portsmouth, UK) in drops of VECTASHIELD with DAPI to stain the ICM cells blue and the coverslip (cover glass, 8310131, VWR International) sealed with nail varnish.

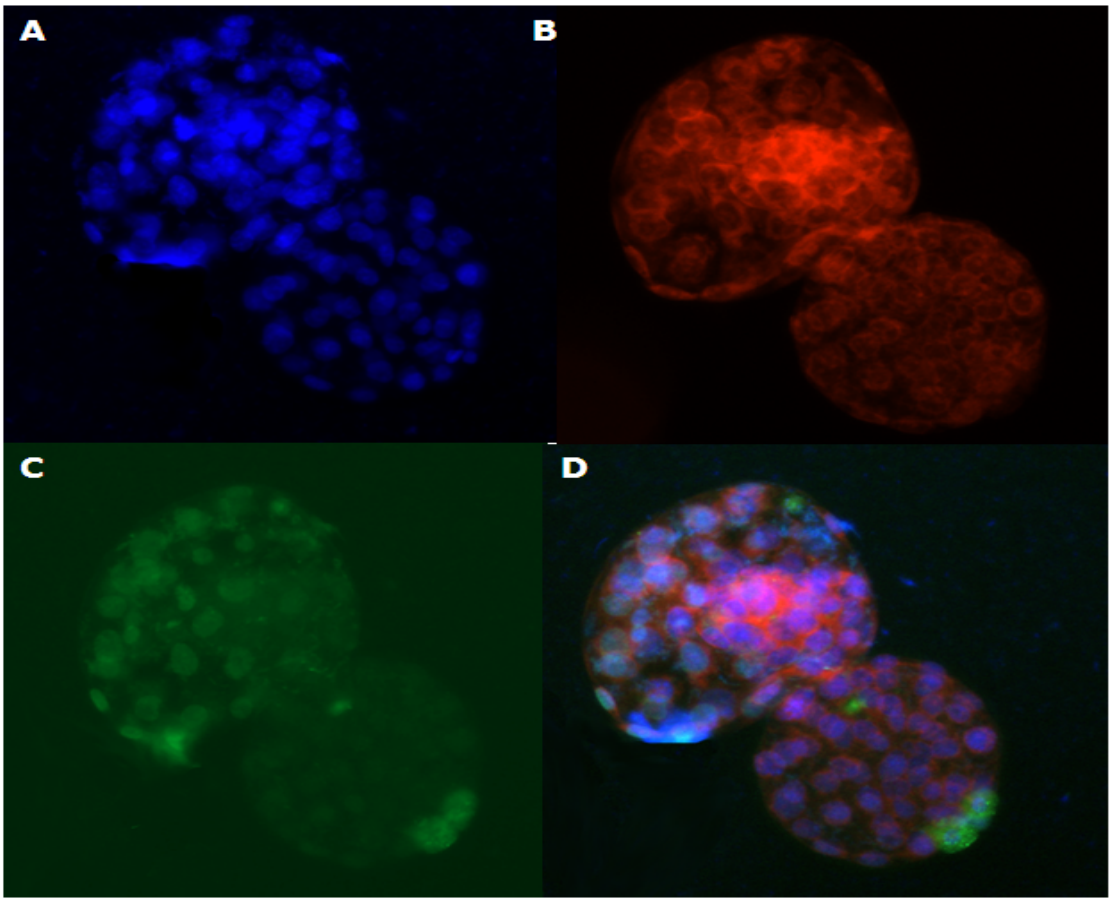

Figure 2.6: Example images from the Volocity of **(A)** DAPI, **(B)** PI, **(C)** Tunel and **(D)** combined stained blastocysts.

2.4.2.3 Fluorescence analysis

Analysis of fixed and differentially stained blastocysts was performed on a fluorescent light microscope (Nikon Eclipse 90i, Nikon Instrument Inc, Derby, UK) using Volocity Software, Version 5.2. Analysis of all three fluorescence stains was performed separately and then combined in a Z stacked image generated of all planes of the blastocysts to enable

quantification of cells in all focal planes (Figure 2.6). Cell counts were performed using the measurements software on the volocity program to count 2D objects and separate touching objects. Cell counts obtained with the software were comparable to those counted manually by visual analysis (Figure 2.7).

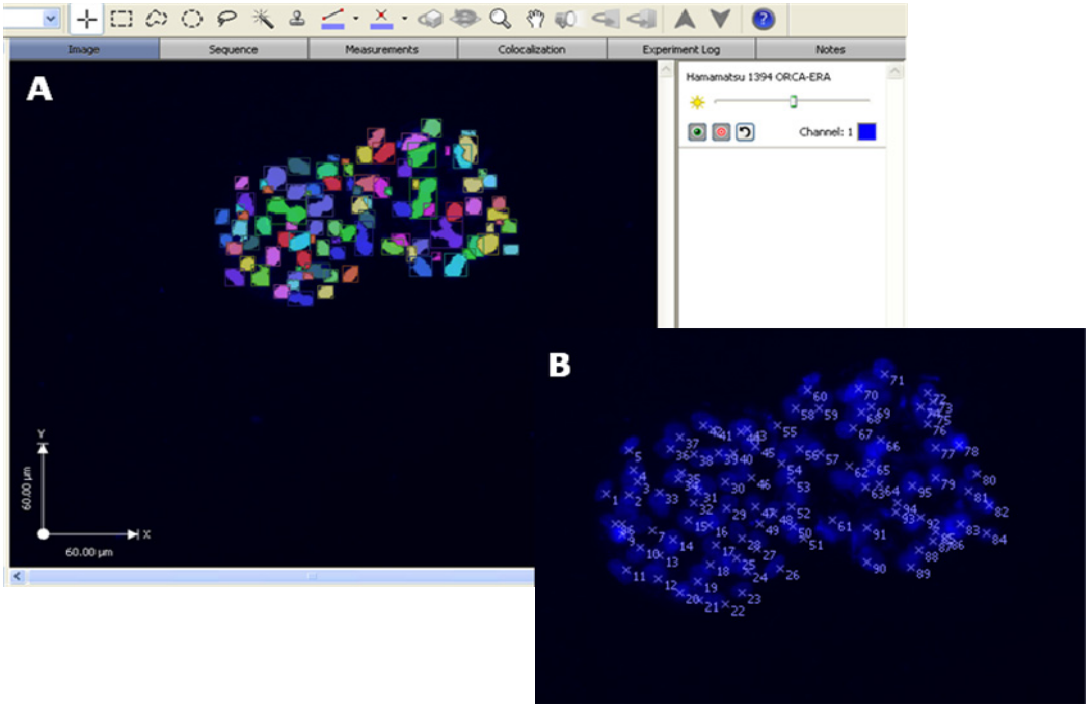

Figure 2.7: Example images from the Volocity software **A** Cell counts calculated automatically by the software. **B** Cell counts manually counted visually.

2.4.3 Gene expression analyses of trophectoderm

Biopsied cell samples were analysed for imprinted gene expression and expression of components of the RAS system.

2.4.3.1 Snap freezing

Due to the limited size of the sample material, biopsied cells were immediately snap frozen in liquid nitrogen to disable any RNase, preventing RNA degradation and aid release of the cells genetic material. Gloves and laboratory coats were used and procedures conducted in a sterile laboratory cleaned with laboratory disinfectant (Trigene Advance,

Medichem International, 023802) to create RNase free conditions. Cells were collected in a minimum final volume of approximately 5µl directly from the G-PGD drop and placed at the bottom of a 0.5ml sterile polymerase chain reaction (PCR) tube (Axygen, California, USA). When all cells were in the tube it was immersed in liquid nitrogen and placed in a -80°C freezer for storage.

2.4.3.2 Total RNA purification

Snap frozen samples were removed from the -80°C freezer and the total RNA and DNA was purified according to the AllPrep™ DNA/RNA Micro kit manufacturers instructions (Qiagen, 820284, West Sussex, UK). Samples were re-suspended in 75µl of Buffer RLT Plus (with added β-mercaptoethanol) before being made to the final volume of 350µl with Buffer RLT Plus and vortexed for 1 minute to homogenise. The lysate was pipetted into an AllPrep DNA spin column, which was placed in a 2ml collection tube. This was centrifuged for 30 seconds at ≥10,000 revolutions per minute (rpm). The AllPrep DNA spin column was discarded and the RNA purified from the flow through in the 2ml collection tube.

350µl of 70% ethanol was added to the flow through and mixed by repeated pipetting. The sample was transferred to an RNeasy MinElute spin column placed in a 2ml collection tube. This was centrifuged for 15 seconds at ≥10,000 rpm and the flow through discarded. Next, 700 µl of Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged at ≥10,000 rpm for 15 seconds to wash the spin column membrane. The flow through was discarded and 500 µl of Buffer RPE was added the RNeasy MinElute spin column and centrifuged for 15 seconds at ≥10,000 rpm. Then, 500 µl of 80% ethanol was added to the column followed by centrifugation at ≥10,000 for 2 minutes. The RNeasy MinElute spin column was then transferred to a new 2 ml collection tube and centrifuged at full speed for 5 minutes with the lid open, to allow the alcohol to evaporate. The column was finally placed in a 1.5 ml collection tube and 14 µl of RNase-free water was pipetted directly into the centre of

the column. This was centrifuged for 1 minute at full speed to elute the RNA. This was stored on ice if cDNA was being produced straight away or stored in a -80°C freezer.

2.4.3.3 cDNA production

Subsequent to purification of RNA, cDNA was produced by reverse transcription using the High Capacity RNA-to-cDNA™ Master Mix (4390779, Applied Biosystems). Addition of 4µl of 5X RT Reaction mixture and total RNA (maximum 1µg) to a tube was performed at room temperature and made up to a total volume of 20µl with RNase free water. The reaction was performed in a Techne flexcycler (FTGENE5D, Techgene) according to the following protocol: incubate at 25°C for 5 minutes, 42°C for 30 minutes, terminate at 85°C for 5 minutes and keep at 4°C until use in qPCR.

2.4.3.4 Relative real time quantitative PCR

Quantification of *H19, IGF2, IGF2r, ATR1, ATR2, VEGFA* gene expression in the trophectoderm biospy cDNA samples was performed in singleplex reactions and compared against the endogenous control, 18s (selected based on the results of the geNorm reference gene selection kit by Primer Design)[4], to normalise for starting amount differences. Pools of *in vivo* generated blastocysts from 0.5IU hMG superovulated mice were used for the serial dilutions and as a calibrator (reference) for the PCR.[5] The Taqman® PCR reaction mixture (Applied Biosystems) composed of 0.5µl of 20X Taqman Gene Expression Assay (Table 2.3), 5µl of 2X Taqman® Fast Advanced Master Mix (4444963, Applied Biosystems) and 1.5µl of cDNA prepared previously made up to the final volume of 10µl with RNase free water for each replicate was pipetted into the tubes of a 96-well plate (MicroAmp®, 4346906, Applied Biosystems) and subsequently loaded into

[4] See Appendix 4 for results of the geNorm reference gene selection kit.

[5] See Appendix 5 for *in vivo* blastocyst collection method.

the Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). 7500 Software version 2.0.5 (Applied Biosystems) was run on the computer attached to the PCR machine to set the thermocycler profile of: 50°C for 2 minutes, 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. After completion of the PCR run (approximately 40 minutes) the expression levels of the genes were calculated according to the standard curve.

Table 2.3: Taqman® gene expression assays for qPCR

Gene Name	Gene Symbol	Assay ID.	Amplicon Length (bp)	Species
18s ribosomal RNA	Rn18s	Mm03928990_g1	61	Mus Musculus
H19 fetal liver mRNA	H19	Mm00469706_g1	76	Mus Musculus
Insulin like growth factor 2	IGF2	Mm00439565_g1	96	Mus Musculus
Insulin-Like Growth Factor 2 receptor	IGF2r	Mm00439576_m1	64	Mus Musculus
Vascular endothelial growth factor A	Vegfa	Mm01281449_m1	81	Mus Musculus
Angiotensin II, type 1 receptor associated protein	ATR1	Mm00507771_m1	59	Mus Musculus
Angiotensin II receptor, type 2	ATR2	Mm01341373_m1	86	Mus Musculus

2.5 STATISTICAL ANALYSES

All data was tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests using SPSS version 16 (IBM Software Services, Hampshire, UK). Normally distributed data was expressed as mean±SEM whereas non-parametric data was presented as median (inter-quartile range). Statistical relevance was set at $P<0.05$ for all analyses in this thesis. Statistical analyses of development data was performed using a non-parametric general analysis of variance (Kruskal-Wallis test) on SPSS having confirmed the data was not normally distributed or transformable by logarithm or square root. See experimental chapters 3 and 4 for descriptions of the statistical variables used. The data was analysed according to treatment group and all interactions were included between different groups. Statistical analyses of the blastocyst triple staining data was performed on SPSS for cell count data and also Graph Pad prism 6 (GraphPad Software, California, USA) for chi squared test for associations for the analysis of proportional data. See the experimental chapters 4 and 5 for more specific descriptions of the statistical models used. Statistical analyses of the ovarian histology data was performed using SPSS. All the data was non-parametric so significance was tested using the Kruskal-Wallis test (Chapter 3). Gene expression data was assessed using a general analysis of variance (ANOVA) on SPSS after confirming the data was normally distributed or by Kruskal-Wallis test if the data was non-parametric and not transformable. More details can be found in the relevant chapters (5 and 6). To analyse weight and organ trajectory in progeny born from the different treatments, a multiple linear regression analysis was performed by using the general linear model in SPSS. See chapter 6 for more details.

CHAPTER 3: THE EFFECT OF OVARIAN STIMULATION ON OOCYTE QUALITY

3.1 INTRODUCTION

The effect of the use of exogenous gonadotrophins on embryo development has been particularly well characterised in mice stimulated with PMSG and hCG (Ertzeid and Storeng, 1992; Van der Auwera and D'Hooghe, 2001; Ertzeid and Storeng, 2001). Poor embryo development following ovarian stimulation has been attributed to oocyte quality (Wassarman and Kinloch, 1992). Maternal mRNA stores are accumulated during folliculogenesis and it has been suggested that exogenous gonadotrophins speed up nuclear maturation resulting in insufficient mRNA stores and consequently poor embryo development (Mehlmann, 2005). Embryo developmental arrest commonly occurs at the maternal to embryonic genome switch which occurs at the two cell stage in the mouse (Gardner and Lane, 2005). In Chapter 4 we investigated the efficacy of common human gonadotrophin preparations with different half lives and LH activity (hMG, rFSH and Pergoveris). Alterations in embryo development were evident from the use of different doses of gonadotrophin preparations which varied in terms of their biological clearance rate, FSH isoforms and level of LH-activity. In this mouse model, LH activity was shown to be essential for viable embryo development post fertilisation and based on the known roles of LH in folliculogenesis and the association of perturbed embryonic viability after the different ovarian stimulation regimes, this chapter will address whether these alterations were a consequence of altered folliculogenesis on oocyte quality.

Unfortunately, assessment of oocyte quality is complicated by the fact that its structural integrity must be retained and several alternative indirect markers of oocyte quality have been proposed. In response to the LH surge, the cumulus layer surrounding the oocyte undergoes expansion as a consequence of the synthesis of the glycosaminoglycan

muco-polysaccharide hyaluronan which is excreted by the cumulus cells into the ECM (Mandelbaum, 2000). Cumulus expansion ultimately leads to a breakdown in communication between the somatic cells and the oocyte and through activation of the hyaluronan receptor (CD44) leads to oocyte maturation (Krysko *et al.*, 2008). The cumulus cells are vital orchestrators of oocyte maturation and development (McKenzie *et al.*, 2004). Consequently the cumulus cells may reflect oocyte quality and be used as surrogate for direct analysis of the oocyte itself and as they are separated and discarded during routine IVF anyway are easily accessible (Fragouli and Wells, 2012). As a result of this, analysis of the cumulus cell transcriptome has been proposed as a non-invasive method of assessing and predicting embryo quality (Assou *et al.*, 2006; Assou *et al.*, 2010; van Montfoort *et al.*, 2008). This is especially important in countries where the decision which oocyte should be fertilised and used for treatment needs to be made at the metaphase II (MII) stage. Cumulus expansion is already used as a predictive morphological marker for oocyte selection (Russell and Robker, 2007).

In addition to cumulus markers, markers of follicular health and maturity may be used to assess the effects of ovarian stimulates on oocyte quality. Vascular endothelial growth factor (VEGF), one of the final products of the RAS pathway, is synthesised in theca and granulosa cells and is the main pro-angiogenic factor in the ovary (Agrawal *et al.*, 2002). VEGF is a heparin binding secreted dimeric glycoprotein which is a vascular endothelial cell specific mitogen (Shweiki *et al.*, 1993). VEGF concentrations increase according to the stage of follicular development and are highest in pre-ovulatory follicles. Greater concentrations in the follicular fluid have been associated with oocyte maturity and improved fertilisation rates (Bokal *et al.*, 2005). High VEGF concentrations in the follicular fluid has been correlated with increased vascularisation of the follicle that may translate into better fertilisation rates, improved embryo quality and higher pregnancy rates (Monteleone *et al.*, 2008). VEGF inhibition by VEGF Trap impaired follicle growth, resulting in small poorly vascularised subordinate antral follicles (Herr *et al.*, 2010).

In addition, Myosin heavy chain II (MYHII) promotes angiogenesis by inducing the synthesis of angiotensin II (AngII). MYHII has been shown to be more highly expressed in unstimulated cumulus cells compared to those obtained after COS (de los Santos *et al.*, 2012). This suggests MYHII is down regulated through exogenous gonadotrophin stimulation. AngII has been implemented in oocyte maturation by its stimulatory steroidogenic activity and establishment of follicle dominance through the augmentation of LH activity on the granulosa cells (Ferreira *et al.*, 2011; Siqueira *et al.*, 2012). In the absence of gonadotrophins, AngII can stimulate progesterone, oestradiol and prostaglandin synthesis, in addition to stimulating ovulation and meiotic maturation of oocytes (Acosta *et al.*, 1999; Yoshimura *et al.*, 1992).

The concentration of AngII in the follicular fluid increases immediately prior to ovulation and after exposure to LH and hCG (Acosta *et al.*, 2000). Inhibition of the angiotensin receptor 2 (ATR2) expressed by theca cells with saralasin prevented ovulation (Ferreira *et al.*, 2007). Taken altogether it is suggested AngII has an early role in ovulation by mediating and enhancing the preovulatory LH surge and a later role in luteinisation (Acosta and Miyamoto, 2004). Furthermore AngII has been shown to induce VEGF expression and has been hypothesised to function as a co-regulator with LH in luteal angiogenesis (Herr *et al.*, 2010).

During the early stages of folliculogenesis, the follicle is avascular and receives its nutrients and oxygen through gap junctions (Section 1.2.8). In the later stages of folliculogenesis, a vascular sheath forms on the basal membrane surrounding the follicle to enable the steroidogenic products of the theca cell layer to enter the peripheral blood circulation and to enable cells to receive the required oxygen and nutrients (Cavender and Murdoch, 1988). The granulosa cell layer remains avascular until ovulation, therefore a regulatory mechanism to prevent angiogenesis in the follicle is pertinent (Chuderland *et al.*, 2012).

Pigment epithelium derived factor (PEDF), alternatively known as SERPINF1, is a 50 kDA secreted glycoprotein belonging to the non-

inhibitory members of the serine protease inhibitors (serpin) superfamiliy. PEDF is a natural angiogenesis inhibitor (Dawson *et al.*, 1999). It exerts its anti-angiogenic effect by inhibiting the stimulatory effects of numerous pro-angiogenic factors, the most extensively characterised being VEGF (Stellmach *et al.*, 2001). The PEDF ligand binds the PEDF-R receptor on the cell surface (Notari *et al.*, 2006). PEDF enhances gamma secretase activity resulting in the cleavage of the tyrosine kinase receptor VEGF receptor 1 (VEGFR-1) transmembrane domain, which interferes with VEGF signalling to induce formation of vessels, thus inhibiting angiogenesis (Cai *et al.*, 2006). In addition, PEDF competitively binds VEGFR-2, further inhibiting the pro-angiogenic VEGF activities by reducing the proliferation and migration of proliferative endothelial cells (Zhang *et al.*, 2006). PEDF also upregulates thrombospondin, another anti-angiogenic protein (Guan *et al.*, 2004). PEDF is secreted by granulosa cells and its expression is hormonally regulated.

Increasing doses of oestrogen to mimic its elevated production by granulosa cells in the later gonadotrophin-dependant stages of folliculogenesis causes a gradual decrease in PEDF secretion. Furthermore, exposure to progesterone, mimicking entrance into the luteal phase, drastically suppressed PEDF secretion suggesting that this factor modifies follicular vasculature (Chuderland *et al.*, 2012). At the time of the LH surge, PEDF and VEGF are inversely regulated. High concentrations of hCG elevates VEGF levels in a dose response manner, whilst with incremental doses of hCG PEDF levels decline (Chuderland *et al.*, 2012). It has been proposed that maintaining a balance between these pro and anti-angiogenic factors is critical for regulating angiogenesis (Gao *et al.*, 2001). Based on previous correlations between blood flow and embryo quality, alterations in the vascularity of the follicle may affect patients' treatment outcome and may therefore be suitable predictive markers for oocyte quality in cumulus cell analyses (Bhal *et al.*, 1999; Coulam *et al.*, 1999; Huey *et al.*, 1999; Du *et al.*, 2006; Shrestha *et al.*, 2006).

The objective of this experiment was, therefore, to examine the effect of common human gonadotrophin preparations with different half lives and LH activity (hMG, rFSH and Pergoveris) on the ovarian response to stimulation and oocyte quality. The endpoints measured were cumulus cell expansion, cumulus cell gene expression (*VEGFA*, *PEDF* and *MYHII*), oocyte maturity and ovarian histology.

3.2 METHODS OVERVIEW

Upon reaching a mean weight of 17g at 5-6 weeks of age, mice were randomly allocated to the various treatment groups as described in chapter 2 section 2.1:

Table 3.1: Number of mice in each treatment group

Treatment	Number of technical repeats (n)	Total Number of mice (n)
Un-stimulated	18	72
5IU PMSG	12	49
0.5IU hMG	9	36
5IU hMG	6	23
0.5IU Pergoveris	9	36
5IU Pergoveris	10	41
0.5IU rFSH	4	14
2.5IU rFSH	4	16
5IU rFSH	3	12

The experimental pathways described in this chapter are schematically presented in Figure 3.1. In addition to collecting pronuclear zygotes following natural mating, oocytes were also collected to provide a cumulus cell sample devoid of sperm contamination for PCR analysis.

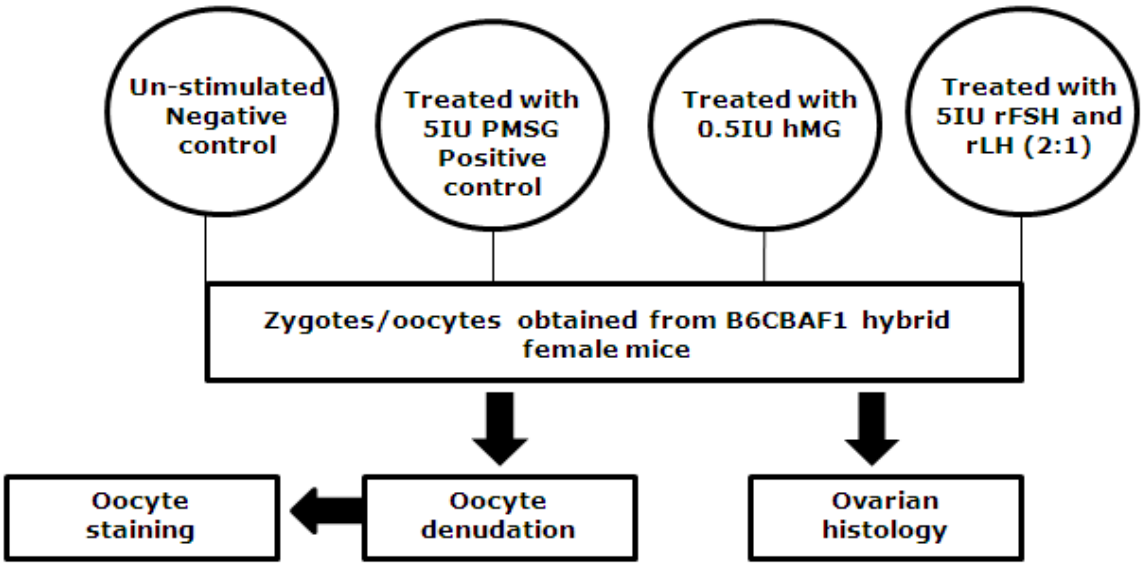

Figure 3.1: Flow chart summarising the experimental design used in this chapter.

3.2.1 Ovarian histology

Ovaries were collected simultaneously to zygotes and fixed in Bouin's solution for 24 hours and stored in 70% alcohol until ready to be processed using a standardised tissue processing protocol and haematoxylin and eosin staining method (Refer to Appendix 7).

3.2.1.1 Ovarian histology analysis

Twenty ovaries per treatment group were randomly selected for analysis. Ovarian sections were analysed for the number of corpora lutea and antral follicles per ovary. The average diameter of corpora lutea per ovary was estimated by calculating the mean of the two maximum widths as depicted in Figure 3.2.

Figure 3.2: Regions of the ovary exhibiting differential staining with tightly packed steroidogenic cells were classed as corpora lutea. The two longest diameters of the corpus luteum were measured and averaged to calculate the mean diameter of *corpora lutea* per ovary.

3.2.2 Cumulus cell morphology

Upon collection of fertilised zygotes, the cumulus expansion status was classified as absent, expanded or dense, as depicted in Figure 3.3.

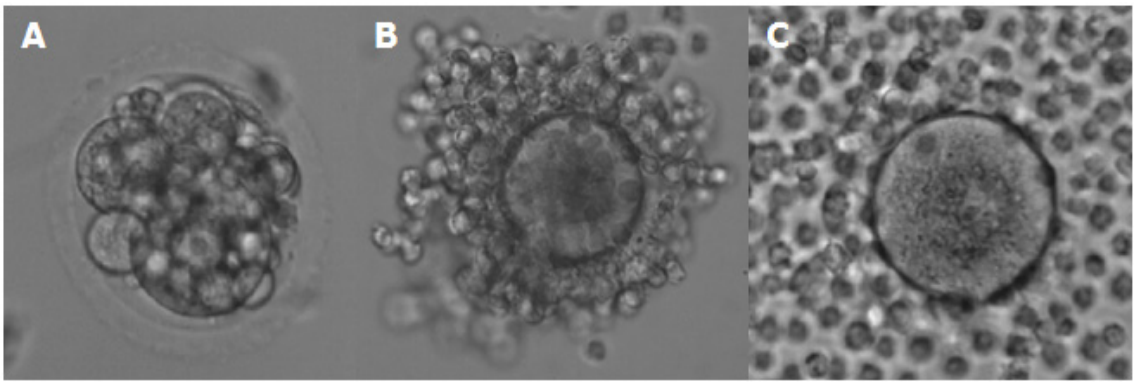

Figure 3.3: Representative images of oocytes **(A)** without cumulus, **(B)** with dense cumulus and **(C)** with expanded cumulus.

3.2.3 Oocyte morphology

Following embryo collection, all collected zygotes were put into culture regardless of maturity status. On Day 2 of *in vitro* embryo culture, those zygotes which had remained un-cleaved were classed as immature or unfertilised oocytes. Figure 3.4 depicts the classifications of oocytes.

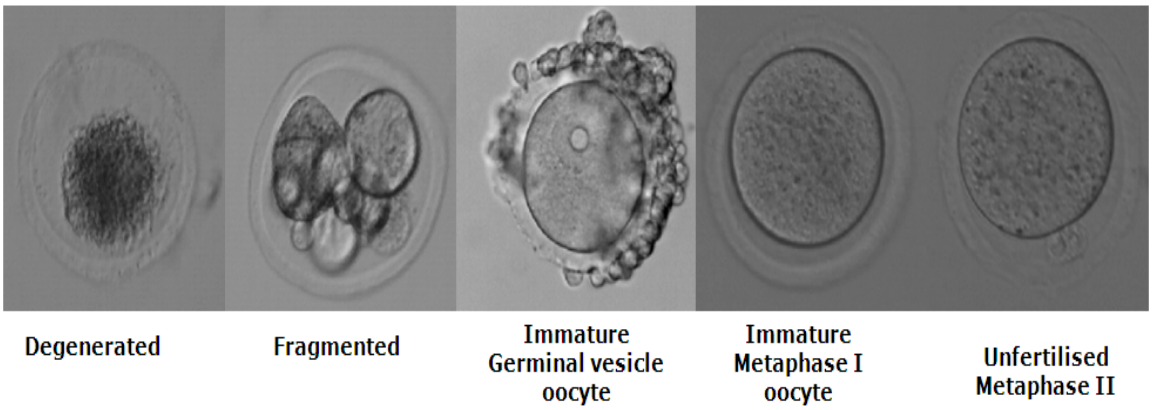

Figure 3.4: Sample images of collected oocytes.

3.2.4 Oocyte collection and denudation

Five to six week old female mice were superovulated with PMSG, 0.5IU hMG and 5IU Pergoveris (n=4 per group), or remained un-stimulated (n=8) and monogamously mated with vasectomised males described in section 6.2.2. Oviducts were collected 24 hours post hCG administration according to the aforementioned method in section 2.3.1. Individual oocytes were separated from the COCs upon release from the oviduct and stored in 5µl drops of G-IVF media (Vitrolife)[6] supplemented with 10% HSA under 5ml mineral oil. Oocytes were exposed to hyaluronidase for 10 seconds in 5µl drops under oil and those cumulus cells which remained in the 5µl GMOPS wash drops under oil were immediately snap frozen for later genetic analysis.

[6]See Appendix 1 for media compositions.

3.2.5 Gene expression analysis of cumulus cells

Cumulus cell samples from mature oocytes were randomly selected for each treatment group (n=12 per group). RNA was extracted using the aforementioned AllPrep™ DNA/RNA Micro kit (See section 2.4.3). This was converted to cDNA using the SuperScript® Vilo™ Mastermix according to the manufacturer's instructions. Addition of 4µl of the superscript reaction mixture and 12µl of total RNA to a tube was performed at room temperature and made up to a total volume of 20µl with RNase free water. The reaction was performed in a Techne flexcycler (FTGENE5D, Techgene) according to the following protocol: incubate at 25°C for 10 minutes, 42°C for 60 minutes, terminate at 85°C for 5 minutes and keep at 4°C until use in qPCR. Quantification of *VEGFA, MYHII* and *PEDF* gene expression in the cumulus cell cDNA samples was performed in singleplex PCR reactions and compared against the endogenous control 18s as described in section 2.4.3.4. Pools of cumulus cells from previous studies were used for the serial dilutions and as a calibrator (reference) for the PCR.

Table 3.2: Taqman® gene expression assays for qPCR

Gene Name	Gene Symbol	Assay ID.	Amplicon Length (bp)	Species
Vascular endothelial growth factor A	Vegfa	Mm01281449_m1	81	Mus Musculus
Myosin heavy chain II	Myh2	Mm01332564_m1	102	Mus Musculus
Pigment Epithelium Derived Factor	Serpinf1	Mm00441270_m1	84	Mus Musculus

3.2.6 Statistical analyses

Data was tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests using SPSS version 16 (IBM Software Services, Hampshire, UK). Statistical relevance was set at $P<0.05$ for all analyses. Analysis of the oocyte quality proportional data was assessed by Chi square test using Graph Pad Prism. The ovarian histology data was not normally distributed and could not be successfully transformed by logarithm or square root so was assessed by Kruskal-Wallis test in SPSS. The difference between antral follicle counts (AFC) and number of embryos collected was parametric and analysed by ANOVA. Gene expression data for *PEDF* and *MYHII* was non-parametric and displayed through a box plot. *VEGFA* expression was normally distributed and the means displayed on a bar chart. This data was analysed by Kruskal-Wallis and ANOVA tests in SPSS respectively.

3.3 RESULTS

3.3.1 Ovarian histology analysis

Naturally cycling mice had an average of 6 corpora lutea (CL) per ovary (Table 3.3), which was comparable with ovaries from the positive control PMSG group. Compared to the positive control, ovaries from the 0.5IU hMG, 5IU hMG, 5IU Pergoveris and 5IU rFSH treated mice had significantly more CLs ($P<0.001$). Collected ovaries from the 5IU dose of hMG treated group had a greater increase in CL numbers ($P<0.001$). Higher doses of Pergoveris also resulted in increased numbers of CLs ($P<0.001$), whereas ovaries following treatment with the lower doses of Pergoveris and rFSH were comparable to the controls ($P<0.05$).

Ovarian stimulation led to an almost 2 fold increase in CL diameter in collected ovaries compared to un-stimulated control ovaries ($P<0.001$) and there were no differences between the regimes ($P>0.05$) except for the 2.5IU and 5IU rFSH treated ovaries which had CLs with smaller diameters ($P<0.01$). The mean antral follicle count (AFC) for the un-

stimulated control was 9 per ovary. This was increased by ovarian stimulation with PMSG (P<0.001). Ovarian stimulation with 0.5IU Pergoveris, 5IU Pergoveris and 5IU rFSH resulted in a greater AFC compared to the positive control (P<0.01).

Table 3.3: Summary of median (inter-quartile range) ovarian histology data for the treatment groups (n=20 per group). (* P=<0.05, ** P=<0.01, *** P=<0.001 compared to positive control as tested by Mann-Whitney U test).

Treatment	Number of CL	Mean CL diameter (µm)	AFC	Mean no oocytes ovulated
Natural	6(3)	237(121)***	9(5)	4(2)***
PMSG	5(2)	431(131)	9(4)	10(7)
0.5IU hMG	8(3)***	447(111)	12(7)	11(8)
5IU hMG	13(3)***	444(131)	17(10)	4(5)**
0.5IU Pergoveris	6(3)	435(139)	16(5)**	5(5)***
5IU Pergoveris	9(1)***	407(109)	18(7)**	8(7)
0.5IU rFSH	5(6)	412(143)	11(2)	2(3)***
2.5IU rFSH	8(6)*	400(79)**	8(9)	17(12)
5IU rFSH	13(4)***	387(117)**	20(6)**	12(7)

Figure 3.5 shows representative images of ovarian sections for the different treatment groups. Ovaries following treatment with PMSG, 0.5IU hMG and 5IU Pergoveris showed freshly ovulated follicles in the histology sections. All of the treatment groups demonstrated small follicles within the ovarian cortex (centre) except for the 5IU Pergoveris and 5IU hMG treatment groups. Un-ovulated COCs are evident in the 0.5IU Pergoveris, 5IU Pergoveris and 5IU rFSH treated ovaries, which had more AFC compared to the positive control (P<0.01).

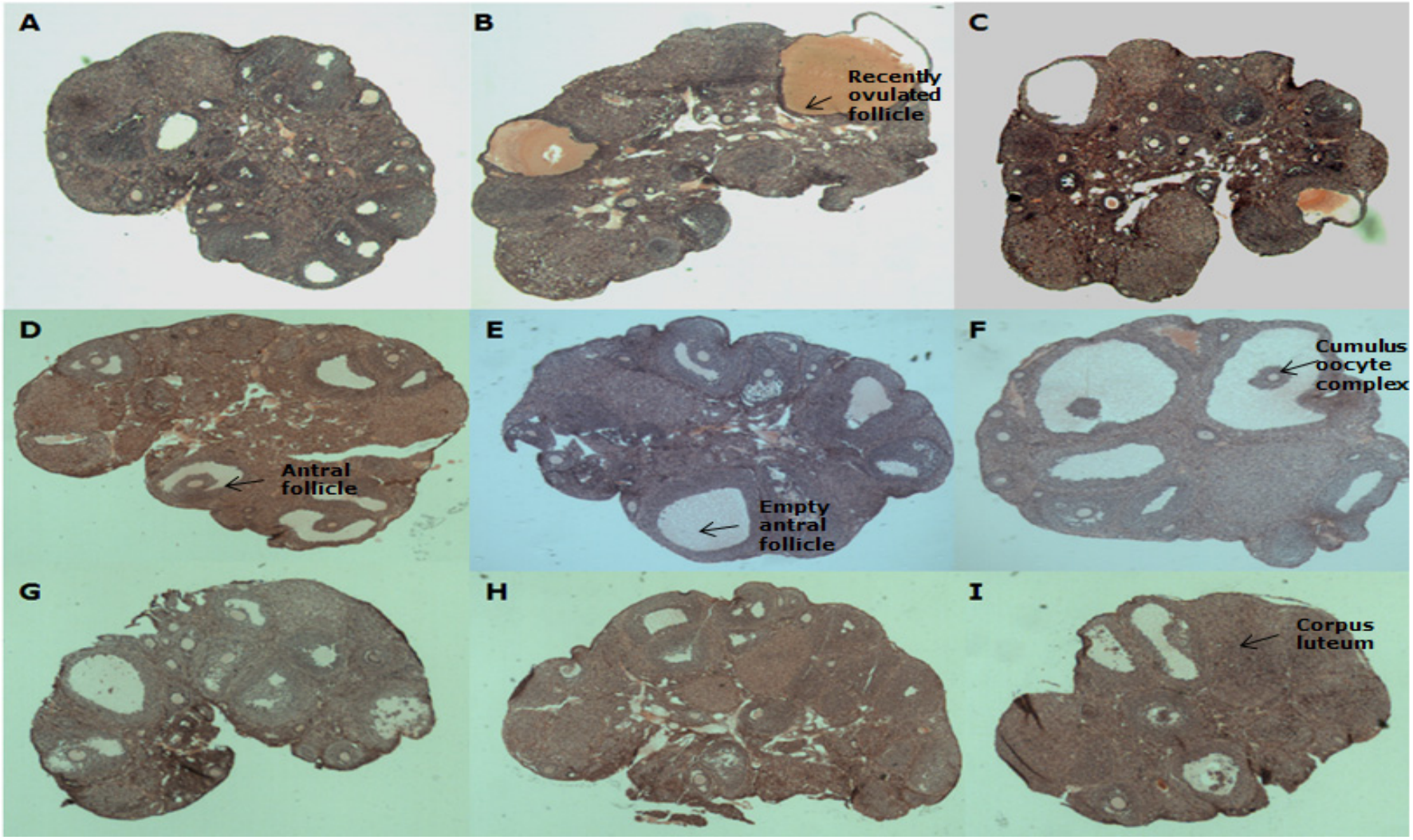

Figure 3.5: Representative images of ovarian histology for **(A)** natural, **(B)** PMSG, **(C)** 0.5IU hMG, **(D)** 5IU hMG, **(E)** 0.5IU Pergoveris, **(F)** 5IU Pergoveris, **(G)** 0.5IU rFSH, **(H)** 2.5IU rFSH and **(I)** 5IU rFSH.

3.3.2 Oocyte quality

3.3.2.1 Immature oocytes

The relative proportion of oocyte and zygote classifications, which failed to cleave, was calculated for each treatment group (Figure 3.6). Naturally ovulated and fertilised zygotes failed to cleave in 10% of cases, whereas all other treatment groups had fewer cleaved embryos on Day 2 ($P<0.001$, see section 4.3.2.1). Un-cleaved zygotes were found in all treatment groups. Seventy seven percent of the natural group oocytes which failed to cleave were unfertilised MII oocytes whereas the remaining 23% had degenerated. Only the ovarian stimulation groups had immature oocytes in their ovulated group and germinal vesicle (GV) stage oocytes were only observed in the rFSH treated group. All treatment groups had a comparable proportion of degenerated zygotes collected compared to the positive control ($P>0.05$), expect for the unstimulated control and 5IU Pergoveris groups ($P<0.05$ and $P<0.01$ respectively).

All the ovarian stimulation regimes resulted in a significant proportion of immature metaphase I (MI) and fragmented oocytes compared to the natural control ($P<0.001$). Fragmentation was only observed following treatment with exogenous gonadotrophins ($P<0.001$). The 0.5IU Pergoveris and rFSH groups had less fragmented oocytes compared to the positive PMSG control ($P<0.001$ and $P<0.01$), however this may be due to there being higher proportions of MI oocytes following stimulation with these preparations ($P<0.001$ and $P<0.05$). Treatment with 5IU Pergoveris resulted in the lowest proportion of metaphase I oocytes ($P<0.01$).

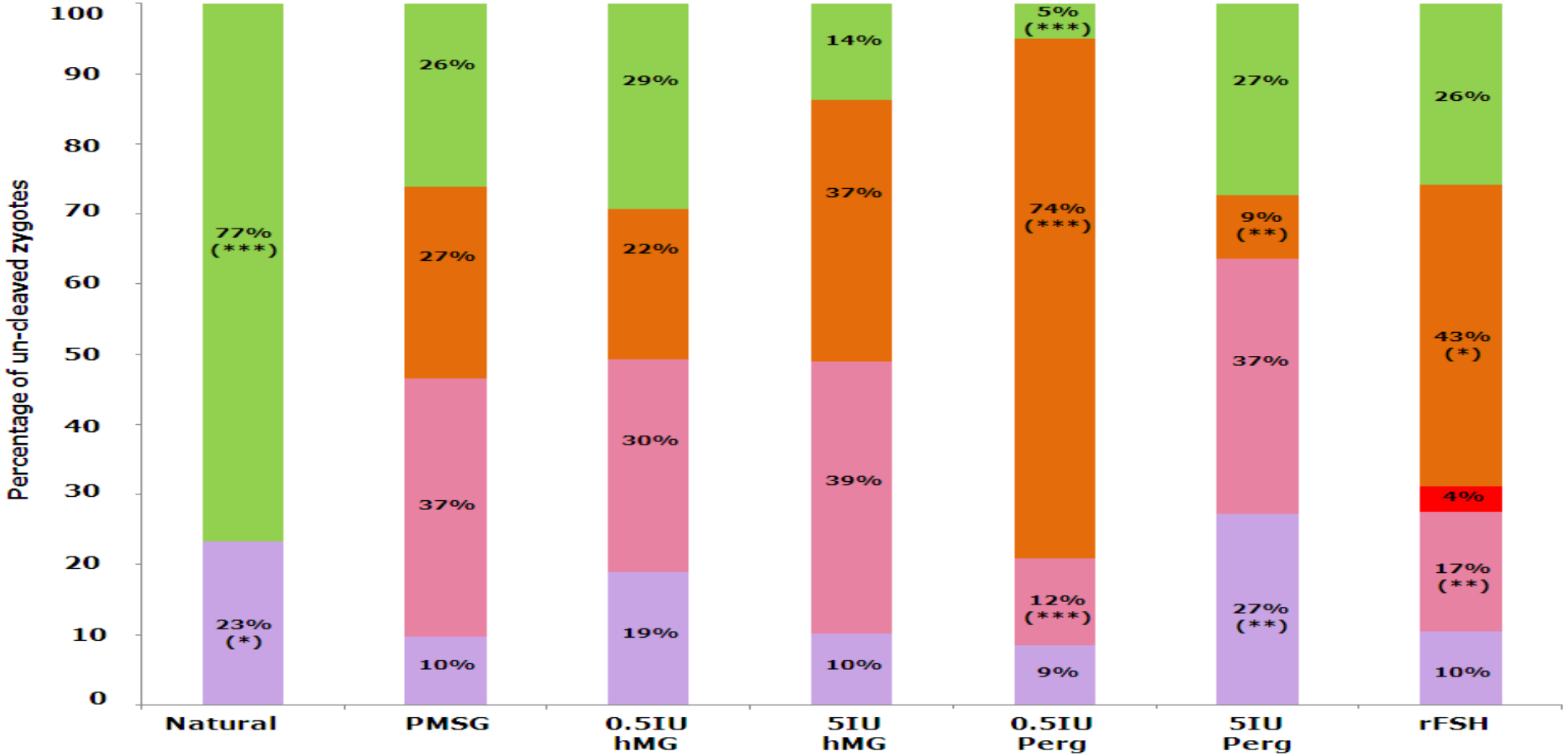

Figure 3.6: Proportion of the total number of uncleaved zygotes for the natural (n=112), PMSG (n=478), 0.5IU hMG (n=499), 5IU hMG (n=109), 0.5IU Pergoveris (n=289), 5IU Pergoveris (n=47), and combined rFSH (n=206) treatment groups classified as degenerated (purple), fragmented (pink), Germinal vesicle (red), metaphase I (orange) and unfertilised metaphase II (green). (*** P<0.001, ** P<0.01, * P<0.05 compared to positive PMSG control as tested by chi square test for associations).

3.3.2.2 DAPI staining of oocytes

Fragmented oocytes had their DNA dispersed throughout all the visible fragments (Figure 3.7A). Both the germinal vesicle and metaphase I oocytes displayed the expected DNA appearance of a nucleus and chromatids undergoing anaphase respectively. Figure 3.7D-E demonstrates oocytes that were unable to extrude the second polar body successfully. Furthermore, Figure 3.7F shows an oocyte that was unable to complete syngamy after fertilisation.

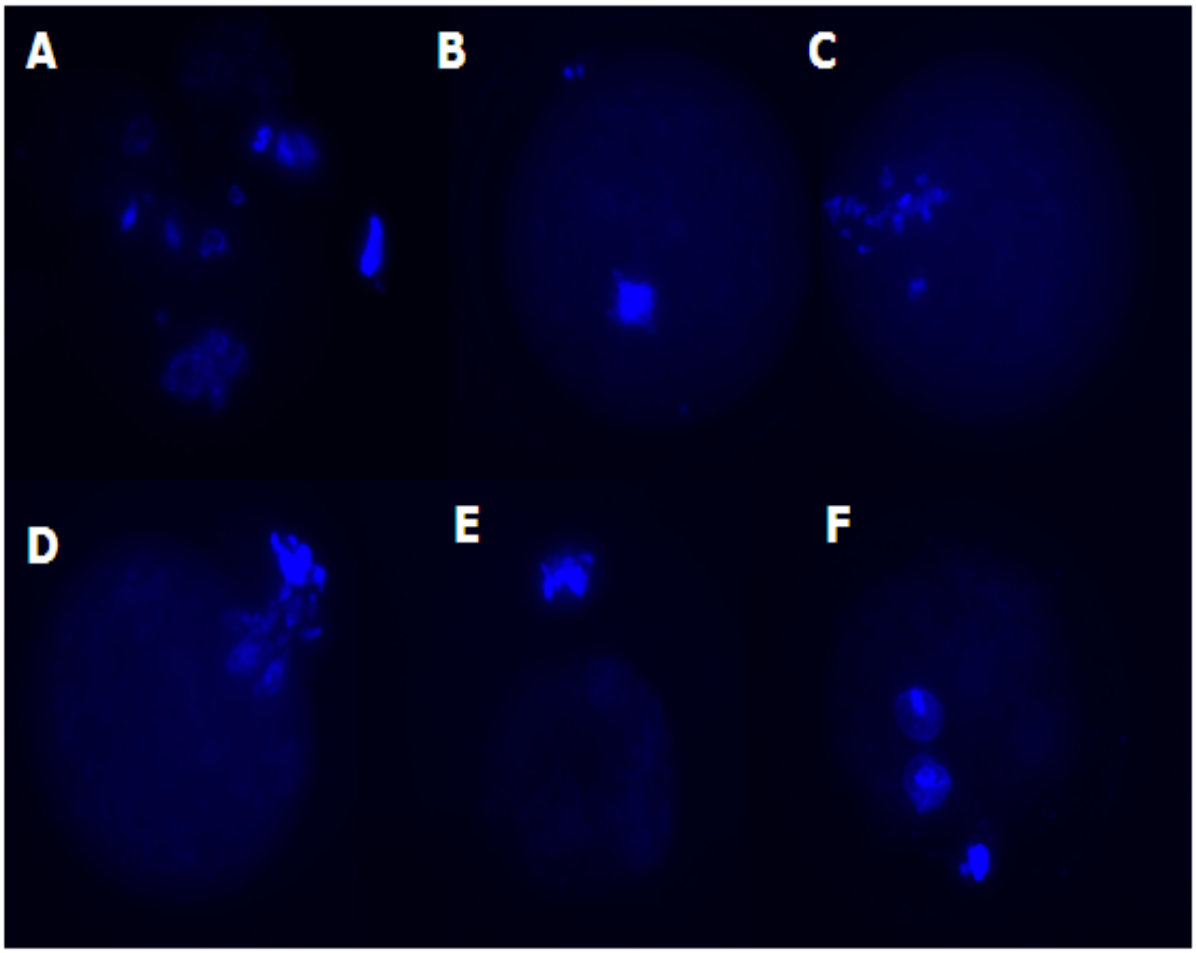

Figure 3.7: DAPI stained immature oocytes which were microscopically classified as **(A)** fragmented, **(B)** germinal vesicle stage, **(C)** metaphase I stage, **(D-F)** metaphase II stage.

3.3.3 Cumulus expansion

In comparison to the un-stimulated control group, which had 100% of ovulated oocytes with expanded cumulus cells, the ovarian stimulation groups had fewer oocytes with intact cumulus cells (Figure 3.8, $P<0.001$). Oocytes densely surrounded by cumulus cells were not evident in the natural group, but the relative proportions of those ovulated in the ovarian stimulation groups were low and comparable to the control ($P>0.05$). Oocytes ovulating without a cumulus were only apparent in the ovarian stimulation groups and significantly more so in the 5IU hMG treatment group compared to the positive control ($P<0.001$). Oocytes which did not have any cumulus associated with them were associated with fragmentation and degenerated morphology. Those with a heavy cumulus mass were difficult to denude with hyaluronidase and in the majority of cases, were immature oocytes.

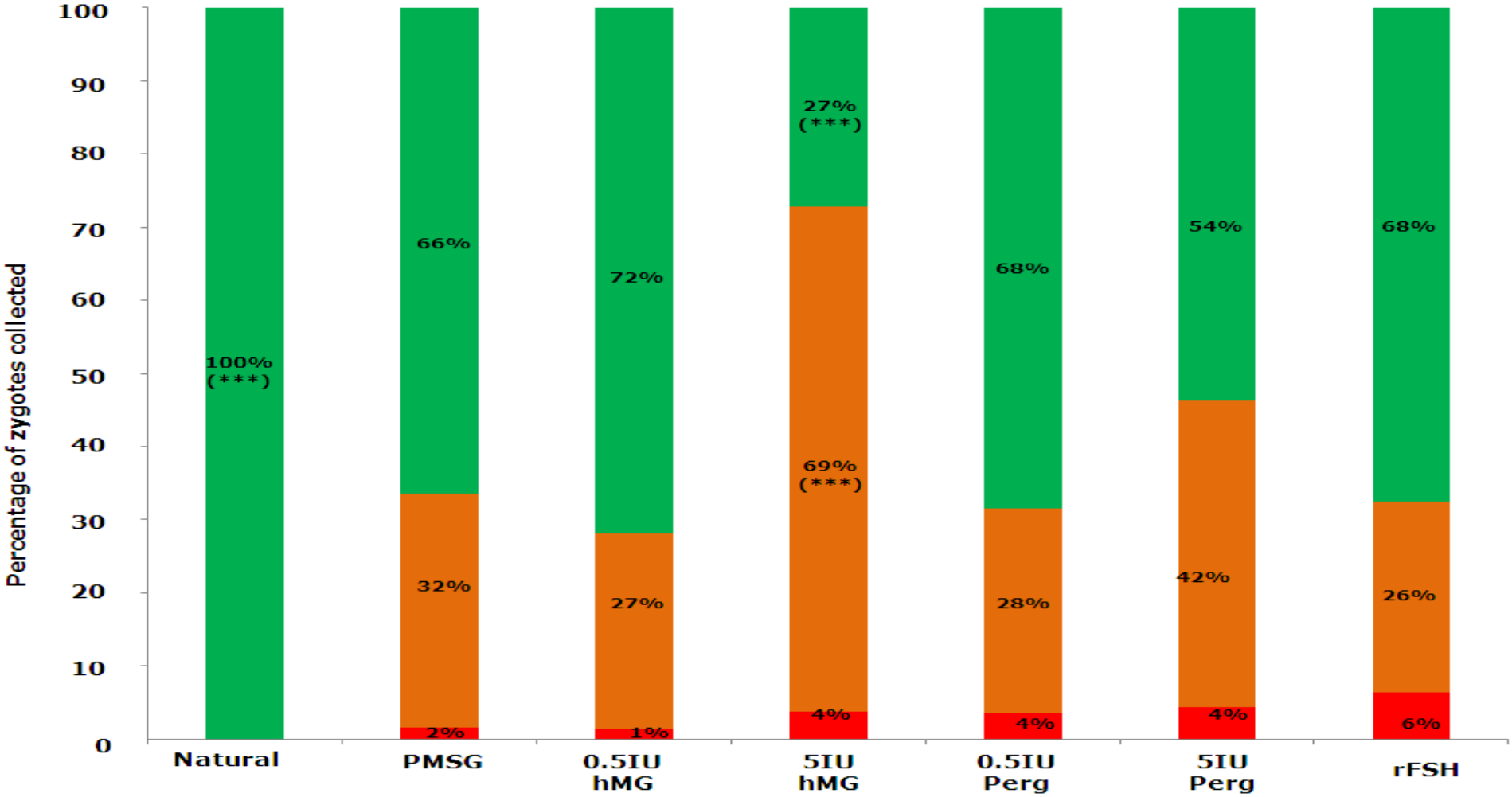

Figure 3.8: Proportion of oocytes cumulus cells for the natural (n=256), PMSG (n=653), 0.5IU hMG (n=420), 5IU hMG (n=184), 0.5IU Pergoveris (n=273), 5IU Pergoveris (n=546), 0.5IU rFSH (n=84), 2.5IU rFSH (n=176) and 5IU rFSH (n=185) treatment groups classified as dense (red), absent (orange) and expanded (green) for the entire group ovulated. (*** P<0.001, *P<0.05 compared to positive PMSG control as tested by chi square test for associations).

3.3.4 Cumulus gene expression data

3.3.4.1 VEGFA gene expression

VEGFA expression in cumulus cells removed from naturally ovulated oocytes had a relative median fold difference of *VEGFA* of 1.61±0.26 (Figure 3.9). Cumulus cells from stimulated mice had nearly a 0.5 fold increase in *VEGFA* mRNA transcripts compared to controls however these trends were not statistically significant (P>0.05) and there were no differences between regimes (P>0.05).

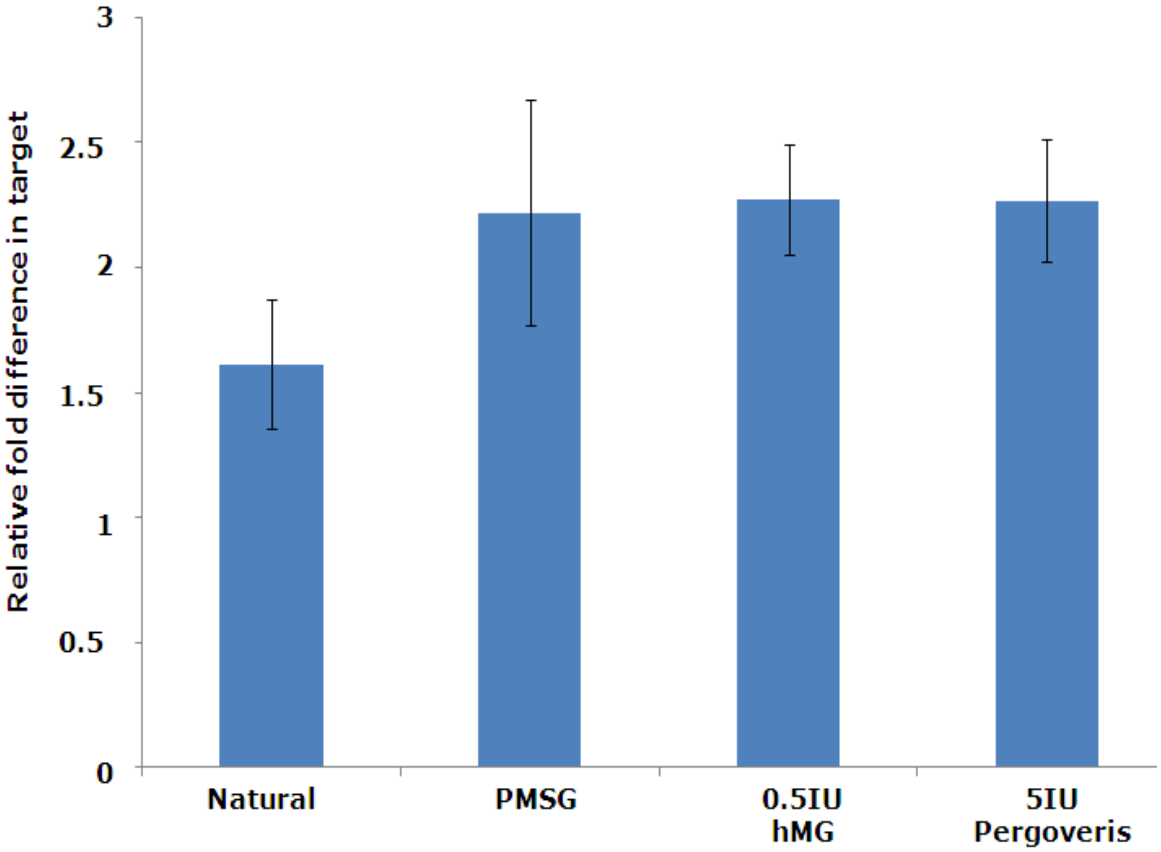

Figure 3.9: Mean ± SEM relative expression of *VEGFA* in natural, PMSG, 0.5IU hMG and 5IU Pergoveris treatments (n=11 per group) No statistical significance found with ANOVA.

3.3.4.2 PEDF gene expression

The relative median fold difference of *PEDF* gene expression in the natural group was 13.94 with an inter-quartile range of 29.65. The PMSG treated group had significantly less mRNA transcripts with a 16 fold decline compared to control (P<0.001, Figure 3.10). Treatment with 0.5IU hMG resulted in a 6.5 fold reduction in *PEDF* gene expression levels (P<0.05), whereas the 5IU Pergoveris group did not differ (P>0.05).

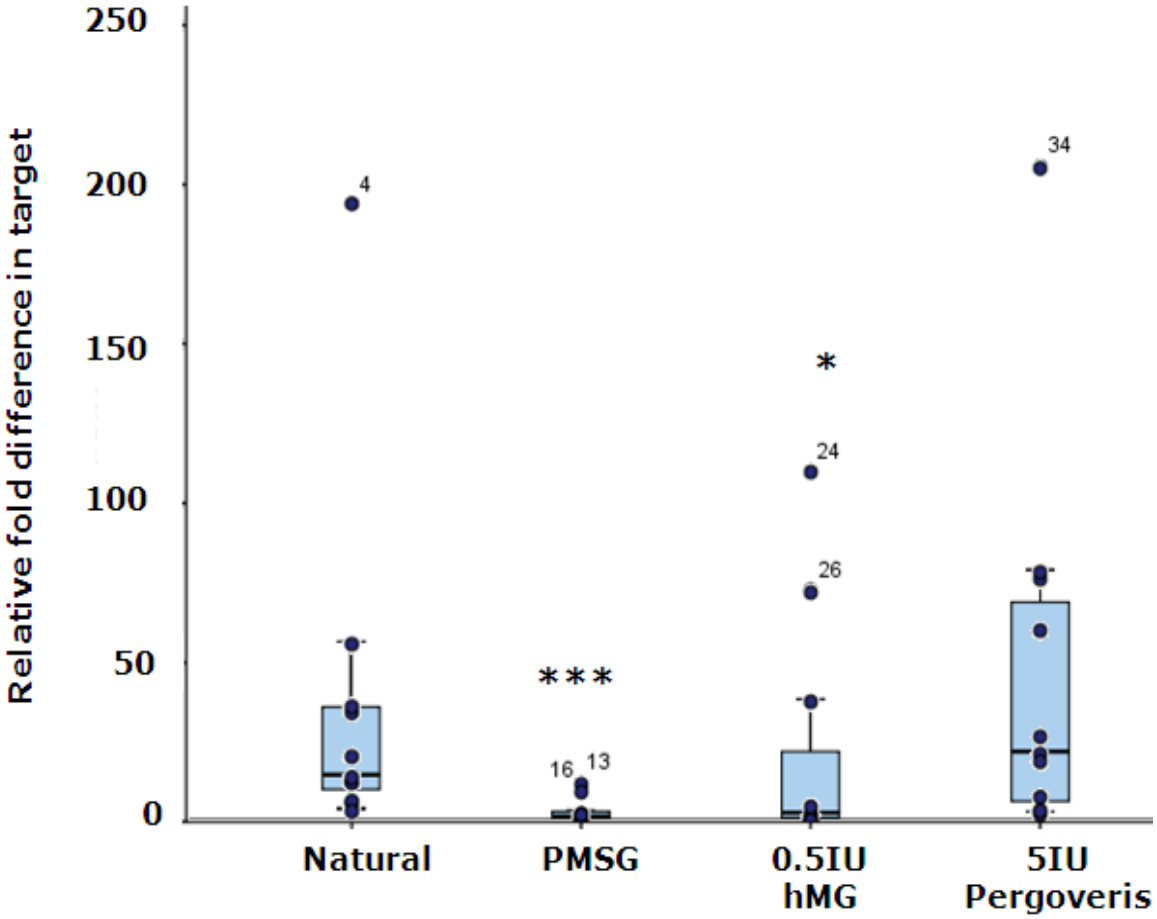

Figure 3.10: Median relative expression of *PEDF* in natural, PMSG, 0.5IU hMG and 5IU Pergoveris treatments (n=11 per group). Bars show inter-quartile range. (* P<0.05, *** P<0.001 compared to natural control as tested by Mann-Whitney U test).

3.3.4.3 MYHII gene expression

MYHII gene expression in the natural group was 1.92 with an inter-quartile range of 2.32. Cumulus cells from mice treated with PMSG had a 2.5 fold decline in gene expression (P<0.05, Figure 3.11).The 0.5IU hMG and 5IU Pergoveris groups demonstrated a 2-fold decrease in gene expression compared to the natural group. This trend however was not statistically significant (P>0.05).

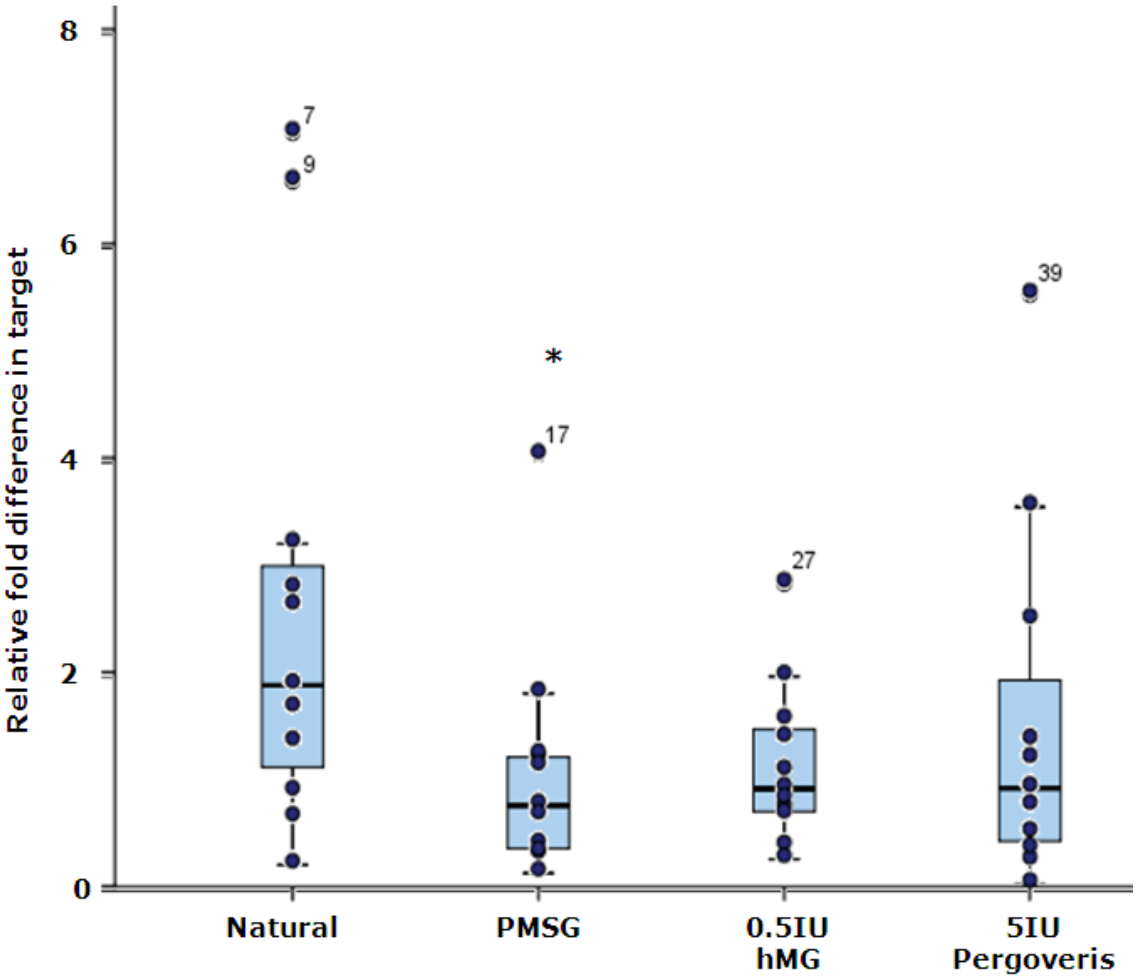

Figure 3.11: Median relative expression of *MYHII* in natural, PMSG, 0.5IU hMG and 5IU Pergoveris treatments (n=11 per group). Bars show inter-quartile range. (* P<0.05 compared to natural control as tested by Mann-Whitney U test).

3.4 DISCUSSION

In this study, it was hypothesised that ovarian stimulation would result in the ovulation of developmentally compromised oocytes due to an effect of exogenous gonadotrophins on oocyte development and quality during folliculogenesis. The results obtained suggest that superovulation does have an impact during folliculogenesis, on oocyte morphology, cumulus expansion and cumulus gene expression.

3.4.1 Ovarian stimulation and oocyte quality

The superovulation groups had a significantly higher proportion of ovulated and fertilised zygotes that did not complete the first cell division, when compared to un-stimulated controls. Previous studies have provided some information regarding the proportion of immature or abnormal oocytes recovered, which is in accordance with the figures in our study (Kanter *et al.*, 2004; Ozgunen *et al.*, 2001; Muñoz *et al.*, 1995). However, the classification of the morphological appearance of these un-cleaved zygotes has not been previously performed. Degenerated oocytes were observed in all treatment groups potentially due to the invasive nature of collecting and handling the zygotes, as was previously reported (Roustan *et al.*, 2012). Poor embryo development after ovarian stimulation has been hypothesised to be due to accelerated development of gonadotrophin responsive follicles so that normal meiotic and cytoplasmic maturations do not occur. Our results support this hypothesis by the observation of immature metaphase I (MI) oocytes only within the stimulated ovulated groups.

All ovarian stimulation regimes led to an increase in the number of immature MI oocytes at the expense of mature MII oocytes. Germinal vesicle stage oocytes were present only in the 0.5IU rFSH treatment group, suggesting low doses of rFSH was not sufficient to remain above the threshold level required for complete development of the follicle. Fragmentation was a phenomenon which was only observed in the ovarian

stimulation groups and has previously been shown to not be compatible with continued embryo development upon fertilisation (Veeck, 1988). Fluorescent analysis of these embryos showed DNA to be dispersed throughout the various cytoplasmic clusters of the oocyte, thus leading to arrest. The proportion of fragmented oocytes increased parallel to dosage in the Pergoveris groups. Furthermore the hMG groups regardless of concentration and PMSG had the highest rates of oocytes with fragmentation, suggesting that the presence of LH activity is involved in this superovulation artefact and occurs at lower doses when long acting LH is present. There is clinical evidence which suggests that overstimulation with LH has a negative effect on follicular atresia and luteinisation and consequently, on oocyte development in a dose dependant manner (Hillier, 1994). *In vitro* experiments have illustrated that low doses of LH enhanced steroidogenesis, whilst higher doses enhanced progesterone synthesis but suppressed aromatase activity and inhibited granulosa cell growth (Yong *et al.*, 1992; Shoham, 2002). Elevated LH concentrations have also been associated with poor fertilisation and implantation rates and adverse pregnancy outcomes (Hillier, 1994). The evidence therefore, supports the theory of a LH threshold, above which adverse effects on folliculogenesis occur (Tesarik and Mendoza, 2002).

3.4.2 Ovarian histology after gonadotrophin stimulation

Ovarian stimulation resulted in an increase in mean CL diameters in ovaries collected for all the treatment groups. This increase is likely to be due to the continued mitotic expansion of the follicle in response to prolonged exposure to FSH (Oktem and Urman, 2010) and previously shown by other through increased bromodeoxyuridine (BrdU) incorporation in the ovarian surface epithelium (Burdette *et al.*, 2006). The antral follicle count (AFC) was greater than the number of oocytes collected for the majority of the treatment groups. This may potentially be due to errors in tissue preparation or analysis. These errors were minimised by every 50μm sections being assessed and one individual

performing all the analyses to insure every CL was investigated once. The results may indicate that a proportion of the pre-ovulatory follicles did not ovulate Therefore, the observation that a number of antral follicles failed to ovulate after superovulation in our study may reflect the functionally different states of the follicles, which may have responded differently to the exogenous gonadotrophins in accordance with their developmental stage (McNatty *et al.*, 2010).

LH and hCG has previously been shown to lead to down regulation of the LH receptor on granulosa cells in humans (Jeppesen *et al.*, 2012) and rats (Rao *et al.*, 1977; Peng *et al.*, 1991), in addition to increasing androgen and cAMP production (Hillier, 1994). The incorporation of hCG into hMG, may potentially at higher doses exacerbate these effects during ovarian stimulation. This would explain why high concentrations of hMG resulted in basal numbers of oocytes ovulating. Higher doses of hMG also led to the largest proportion of ovulated oocytes lacking cumulus cells, which has been shown to result in non-viable oocytes due to the inability to complete meiosis (Carabatsos *et al.*, 2000). This may explain why the high dose hMG group yielded a large proportion of immature and poor quality oocytes, which may subsequently effect embryonic development.

3.4.3 Relationship of cumulus factors with embryo quality

Cumulus expansion is a morphological marker currently used to predict oocyte competency for use in ART (Russell and Robker, 2007). Our study demonstrated that oocytes which did not have any cumulus associated with them were mostly fragmented and of poor quality and those with a heavy cumulus mass were difficult to denude with hyaluronidase and generally immature. Therefore, neither were compatible with further embryonic development as previously reported (Mandelbaum, 2000). All oocytes ovulated without stimulation had full cumulus expansion, which occurred at varying reduced levels in the stimulation regime groups.

Although the expression of the pro-angiogenic factor *VEGFA* in cumulus cells from un-stimulated and superovulated oocytes were comparable, levels of *PEDF* were reduced for both PMSG and 0.5IU hMG treatments. *PEDF* is a natural angiogenesis inhibitor (Dawson *et al.*, 1999) and the balance between these pro- and anti- angiogenic factors has been hypothesised to be critical for regulating angiogenesis (Gao *et al.*, 2001). *In vitro* studies indicated that elevated levels of oestrogen caused a gradual decline in *PEDF* expression (Chuderland *et al.*, 2012). Both the PMSG and hMG preparations contain long acting LH bioactivity. LH in combination with FSH stimulates oestrogen production as discussed in section 1.2.6.1, which may explain the decline in *PEDF* expression in these regimes. In addition, the ovarian histology data showed the number of oocytes ovulated per ovary was significantly elevated for the PMSG and hMG group, suggesting that there may have been more oestrogenic follicles within these treatment groups. The Pergoveris treatment groups may have been unaffected due to the low half life of the recombinant LH in this preparation.

Previous reports suggested that increased angiogenesis in the follicle is associated with improved embryo development (Bhal *et al.*, 1999; Coulam *et al.*, 1999; Huey *et al.*, 1999; Du *et al.*, 2006; Shrestha *et al.*, 2006). Angiogenesis is controlled by multiple factors and expression of *MYHII* was reduced in cumulus cells from oocytes ovulated after treatment with PMSG as indicated in a previous study (de los Santos *et al.*, 2012). This factor promotes angiogenesis by inducing synthesis of AngII, which is associated with steroidogenesis, meiotic maturation and ovulation (Ferreira *et al.*, 2011; Siqueira *et al.*, 2012); therefore reduced expression may impair oocyte quality. Although marginally reduced in all ovarian stimulation regimes, *MYHII* expression is only significantly diminished following PMSG treatment which may explain the more perturbed embryo development in this treatment group (Chapter 4).

Inadequately vascularised follicles have previously been shown to produce oocytes with spindle defects which the authors attributed to the hypoxic conditions which may hinder oxidative metabolism and consequently lower the intracellular pH (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997). Fluorescent microscopy analysis of the metaphase II oocytes, which failed to divide in our study, demonstrated that the majority of the oocytes did have some developmental abnormalities. These oocytes showed inappropriate division of the genetic material or failure to complete syngamy, were the visible causes for developmental arrest and the timing of this arrest prior to ZGA suggests it may be an underlying oocyte quality issue, potentially spindle related (Pesty *et al.*, 2007). Only un-cleaved oocytes from ovarian stimulation regimes exhibited this morphology. Visualising the mitotic spindle by immuno-histochemical staining of the cytoskeletal structure by injection of fluoroscein-labelled α-tubulin into un-cleaved metaphase II oocytes may provide more evidence for any spindle defects (Paddy *et al.*, 1996).

3.4.4 Conclusion

The results of this study indicate that the quality of oocytes obtained following ovarian stimulation of mice with human gonadotrophin preparations is compromised as has previously been reported for PMSG. The evidence suggests in terms of oocyte and cumulus characteristics, that there are no differences in oocyte quality between the different ovarian stimulation regimes, although this quality is significantly reduced in comparison to the unstimulated control. These studies have confirmed the observation that ovarian stimulation with gonadotrophins results in fragmented oocytes, although the exact mechanism resulting in this response remain unclear (Hillier, 1994). Angiogenesis may also be involved in determining oocyte quality as demonstrated by reduced expression of *MYHII* in cumulus cells from gonadotrophin treated mice. Future studies described in the following chapters of this thesis will examine the effects of ovarian stimulation on embryo development, in addition to molecular, *in vivo* and post partum developmental consequences of the differences in oocyte and embryo quality induced by these different stimulation regimes.

CHAPTER 4: THE EFFECT OF OVARIAN STIMULATION ON EMBRYO QUALITY

4.1 INTRODUCTION

Pregnant mare's serum gonadotrophin (PMSG) was the first preparation used to induce ovarian stimulation and its application in rodents, such as the mouse and other mammals is still widely utilised (Lunenfeld, 2004). PMSG is derived from the endometrial cups of pregnant mares and although similar to hCG in terms of molecular structure, has an extended half life. PMSG also has both FSH and LH activity so it can function as a stimulator and ovulation inducer (Chopineau *et al.*, 1997). PMSG was originally used in humans to recruit multiple follicles (Beall and DeCherney, 2012). Attempts to induce ovulation after follicle development with PMSG were inconsistent (Lunenfeld, 2004). The use of PMSG was further hampered by inter species differences which were causing the formation of antibodies to the exogenous gonadotrophin, which is some cases led to a cross reaction with endogenous FSH and LH (Macklon *et al.*, 2006). In addition, the approval of the use of the animal derived PMSG in human fertility management was revoked in 1972 (Lunenfeld, 2004). The first gonadotrophin of human origin on the market was human menopausal gonadotrophin (hMG). Like PMSG, hMG provides both FSH and LH bio-activity (Lunenfeld, 2004). Protein purification improved, with the application of immuno-affinity chromatography allowing an increase of the relative amounts of the active FSH ingredient (Huirne *et al.*, 2004), leading to the manufacturing of highly purified hMG (Menopur), which is still in use commonly to date. In this preparation the LH component is removed during the purification of FSH and replaced by 10IU of hCG allowing LH activity constituents to be better controlled (Wolfenson *et al.*, 2005). Due to the six times longer half life of hCG, 10IU provides the equivalent bioactivity of 75IU LH (Huirne *et al.*, 2004). More recently the advent of recombinant DNA technology has led to the production of recombinant gonadotrophins (Balen *et al.*, 1999).

Large scale multi centre comparative trials were conducted in the 1990's assessing clinical outcomes with the different pharmaceutical compounds and many independent comparative trials have been performed since. A meta-analysis of these early studies favoured rFSH over uFSH in terms of clinical pregnancy rates (Daya and Gunby, 2000) however this was withdrawn from the Cochrane database due to a conflict of interest and the inclusion of dated trials (Daya and Gunby, 2006). Another meta-analysis from the same period found no difference in cumulative ovulation rates, pregnancy rate, miscarriage rate, multiple pregnancy rate or ovarian hyper stimulation (OHSS) risk when the stimulatory effects of rFSH were compared with its traditional urinary counterpart (Bayram *et al.*, 2001). An updated meta-analysis again showed no disparity in pregnancy rates between hMG, uFSH and rFSH (Al-Inany *et al.*, 2003). The same research group found clinical pregnancy rate and live birth rates in favour of hMG compared to rFSH but only in the IVF patients, including the new highly purified hMG containing hCG as the LH substitution (Al-Inany *et al.*, 2009). Another meta-analysis at the same time found this positive trend for both IVF and ICSI patients with a pooled increase in pregnancy rate of 4% (Coomarasamy *et al.*, 2008).

LH exerts some of its actions on the cumulus cells surrounding the developing oocyte which are involved in bi-directional communication with the oocyte (Matzuk *et al.*, 1990). This is vital to sustain oocyte viability and thus early embryo development. Therefore LH may be an essential component absent in most ovarian stimulation regimes in the endogenous LH levels are fully suppressed (Platteau *et al.*, 2004). Current evidence suggests LH supplementation only benefits certain patient groups such as poor responders and those of advanced maternal age (>35 years) (Mochtar *et al.*, 2007). Despite the LH requirement controversy, superovulation has consistently been associated with numerous disadvantages.

The effect on embryo development with the use of exogenous gonadotrophins PMSG and hCG has been particularly well characterised in the mouse model. Ovarian stimulation using PMSG has been shown to

reduce the proportion of blastocyst formation and a delay in the development to the blastocyst stage both *in vitro* (Ertzeid and Storeng, 1992; Van der Auwera and D'Hooghe, 2001) and *in vivo* (Ertzeid and Storeng, 2001) in mouse models. COS has shown to increase the frequency of chromosomal abnormalities in embryos (Chang, 1977), reduce the total cell number of blastocysts and the number of micro villi on the cell surface (Champlin *et al.*, 1987). Providing a potentially explanation for poor *in vitro* embryo development and the delay observed in implantation (Van der Auwera and D'Hooghe, 2001). Analogous ailments following ovarian stimulation with PMSG have also been reported in other mammalian species such as the hamster (McKiernan and Bavister, 1998), cow (Gonzalez *et al.*, 1994) and sheep (Ryan *et al.*, 1991).

The objective of this experiment was to examine the efficacy of common human gonadotrophin preparations with different half lives and LH activity in mice (hMG, rFSH and Pergoveris). The endpoints measured in this experiment were embryo yield, embryo development, blastocyst grade and blastocyst quality.

4.2 METHODS OVERVIEW

This chapter provides the embryo development results for the *in vivo* fertilised mice which were randomly allocated to the various treatment groups in Chapter 3:

Table 4.1: Number of mice in each treatment group

Treatment	Number of technical repeats (n)	Total number of mice (n)
Un-stimulated	18	72
5IU PMSG	12	49
0.5IU hMG	9	36
5IU hMG	6	23
0.5IU Pergoveris	9	36
5IU Pergoveris	10	41
0.5IU rFSH	4	14
2.5IU rFSH	4	16
5IU rFSH	3	12

The age (5-6 weeks), strain (B6CBAF1), mean weight (18.375g), source (Charles Rivers UK, same breeding colony) and timing of sacrifice and experimental procedures were kept constant throughout the study. After collection (refer to section 2.3.1) all embryos followed the same experimental conditions in this chapter as schematically represented in Figure 4.1.

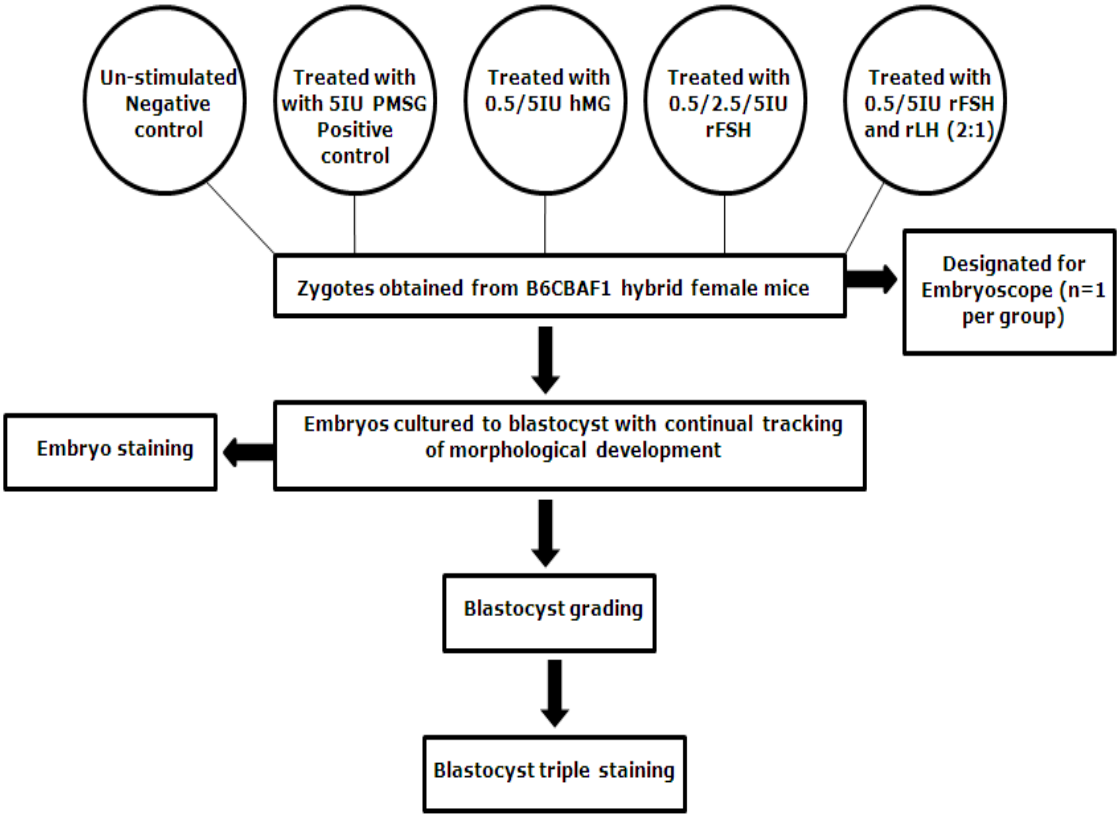

Figure 4.1: Flow chart summarising the experimental design used in this chapter.

Embryos were cultured to blastocyst using the G-1/G-2 sequential culture system described in section 2.3.2. Those embryos which failed to continue development were stained with DAPI to visualise the genetic material (refer to section 2.3.3). On Day 5, blastocysts were graded according to Gardner and Schoolcrafts system (refer to section 2.3.2.2) and triple stained as previously described in section 2.4.2.2. One mouse per treatment group was designated for time lapse imaging using the embryoscope (section 2.3.2.1) to enable comparisons between daily developmental monitoring and time lapse microscopy.

4.2.1 Statistical analyses

Data was tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests using SPSS version 16 (IBM Software Services, Hampshire, UK). Statistical significance was set at $P<0.05$ for all analyses. Statistical analysis of the number of zygotes collected per treatment group was performed using the Kruskal-Wallis test having confirmed the data was not normally distributed and could not be successfully transformed by logarithm or square root. All subsequent statistical analyses in this chapter are made relative to the number of embryos initially collected. This was done to give an indication of successful embryo development based on the initial yield, which in conventional IVF is unknown until post fertilisation and removal of the cumulus cells. Kruskal-Wallis test was also used to analyse developmental data. The proportion of embryos reaching the blastocyst stage was also expressed as actual numbers per mouse and tested by ANOVA having confirmed the data was parametric.

Statistical analyses of the blastocyst triple staining data was performed by Kruskal-Wallis test for the total cell counts and chi squared test for associations to analyse the proportional data. As the number of trophectoderm cells in stained blastocysts was not always clearly discernible, only those embryos that had clear fluorescent images were used for the TE and ICM proportional analysis. Analysis of blastocysts was based on the whole group reaching that stage and the Chi square test was used to statistically assess the results for both morphological stage and grade.

4.3 RESULTS

4.3.1 Embryo yield

Compared to the naturally cycling control group, treatment with 5IU PMSG, 0.5IU hMG, 5IU Pergoveris, 2.5IU and 5IU rFSH significantly (P<0.01) increased the yield of ovulated oocytes (Figure 4.2). Administration of rFSH at the 0.5IU dose resulted in significantly less embryos collected (P<0.001) than naturally ovulating controls, whereas embryo yield for the 5IU hMG and 0.5IU Pergoveris groups did not differ from controls.

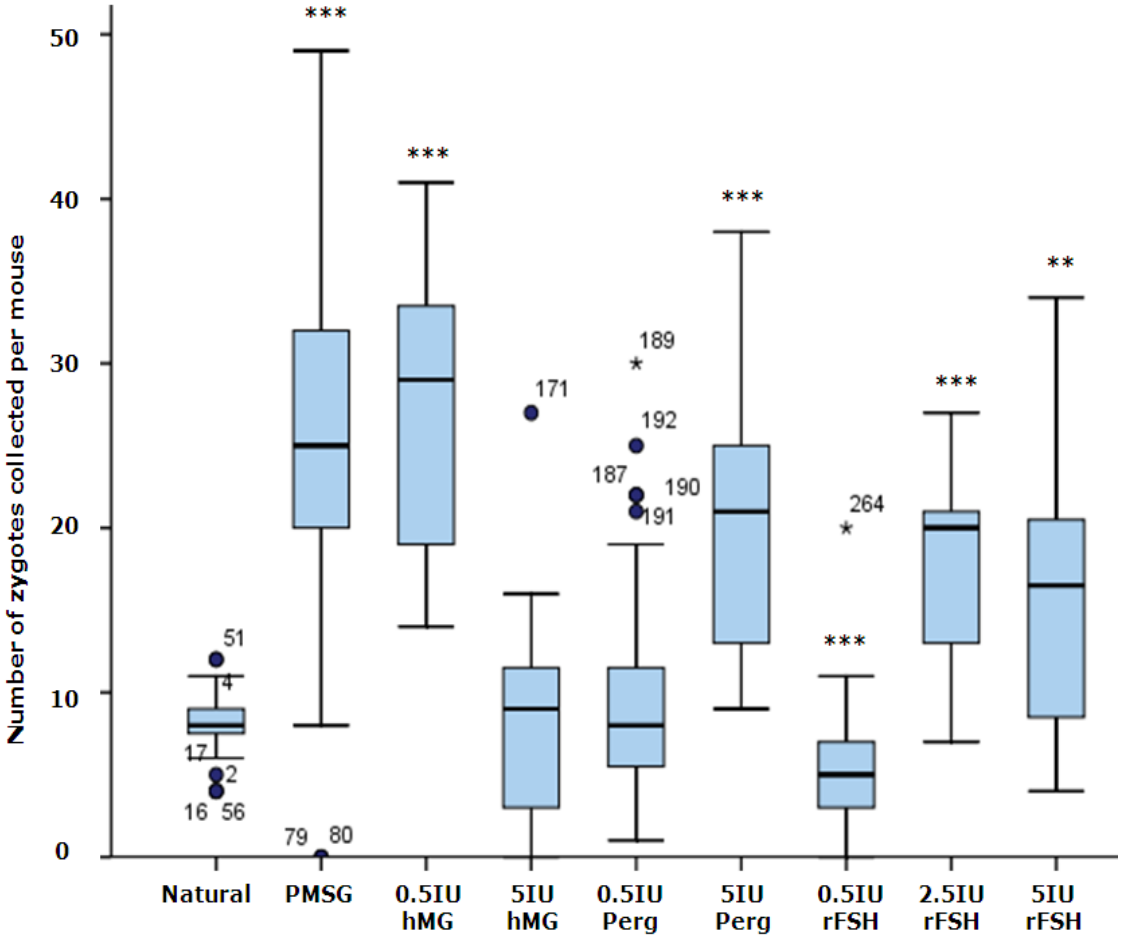

Figure 4.2: Box plot showing median number of zygotes collected for Natural, PMSG, 0.5IU hMG, 5IU hMG, 0.5IU Pergoveris, 5IU Pergoveris, 0.5IU rFSH, 2.5IU rFSH, 5IU rFSH. Bars represent the inter-quartile range. (Significantly different compared to natural *** P<0.001, **P<0.01 as analysed by Mann-Whitney U test).

4.3.2 Embryo quality

4.3.2.1 Proportion developing

Figure 4.3 depicts development as the proportion of embryos reaching the two cell stage, those that continued to divide further past this block and those reaching the blastocyst stage, based on the total number of zygotes collected regardless of maturity. The natural group had 100% cleavage to 2 cells. This was not observed in any of the ovarian stimulation groups. In the natural group all embryos continued developing past the 2 cell block and arrest only occurred prior to the blastocyst phase with one embryo per mouse on average not forming a blastocoel cavity. In all the ovarian stimulation groups significantly less embryos developed to the blastocyst stage when compared with the un-stimulated control (Figure 4.3, $P<0.001$).

PMSG treatment resulted in significantly less embryos undergoing the first cell division ($P<0.001$). Furthermore, a larger proportion of the two cell embryos arrested at this stage, resulting in statistically less embryos continuing development on towards the blastocyst stage ($P<0.001$). The proportion of embryos completing pre-implantation embryo development from the 2 cell stage in the PMSG treatment group was comparable to the natural group ($P>0.05$). The 0.5IU hMG ovarian stimulation group followed a parallel mode of embryo development as those stimulated with PMSG. The 5IU hMG group on the other hand resulted in significant arrest at the two cell stage ($P<0.001$), but as with the 0.5IU hMG and PMSG treatments, of those embryos cleaving past the 2 cell stage, a similar proportion developed to the blastocyst stage.

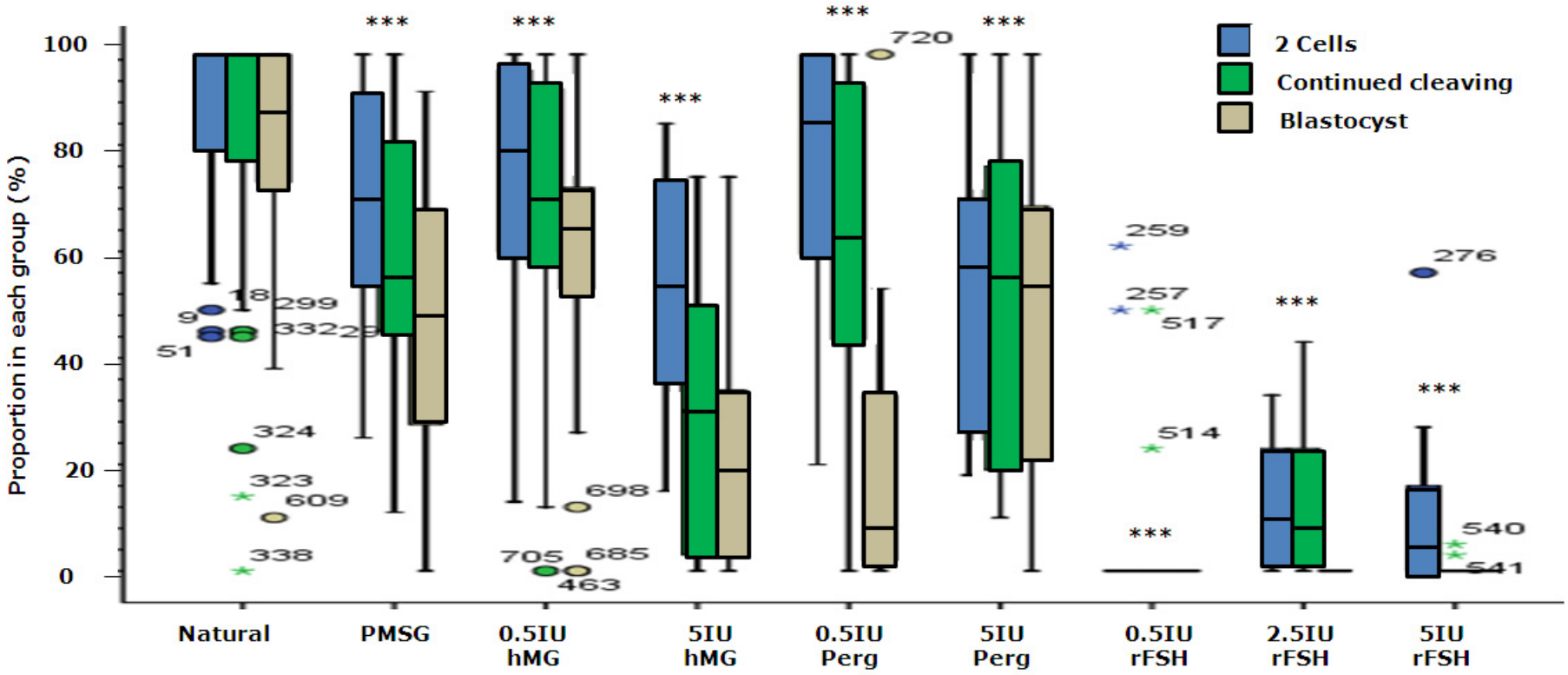

Figure 4.3: Box plot showing the median proportion of embryos cleaving to the 2 cell stage, continuing cleavage past this stage and reaching the blastocyst stage for all the treatment groups relative to number of zygotes collected. All ovarian stimulated groups development was impaired compared to natural (*** P<0.001 as analysed by Mann-Whitney U test). Bars represent the inter-quartile range.

The 0.5IU Pergoveris treatment resulted in less embryos cleaving ($P<0.05$) and as with the other ovarian stimulation groups, resulted in significant arrest of embryo development at the two cell stage ($P<0.001$). In contrast this regime also displayed considerable developmental arrest prior to blastocyst formation ($P<0.001$) resulting in very few blastocysts for further analysis. In contrast the 5IU Pergoveris group, in which, despite having less embryos completing the first cell division ($P<0.001$), development did continue and overcame the two cell block to reach the blastocyst stage.

The rFSH stimulation regimes on the other hand did not yield viable embryos. Embryos from mice treated with 0.5IU rFSH rarely cleaved whereas embryos from mice treated with 2.5IU rFSH had some cleavage and further development whilst embryos from mice treated with 5IU rFSH all arrested at the two cell stage. Throughout the entire study, the 0.5IU and 5 IU rFSH treatments only resulted in 2 and 1 embryos, respectively, reaching the blastocyst stage of development. Further, the blastocysts produced were very early blastocysts, only acquired through extended culture until Day 6. Accordingly, for the remainder of this results section the rFSH treatment groups will not be referred to.

4.3.2.2 Rate of development

On Day 1 of collection zygotes would be expected to be at the one cell pro-nuclear stage as observed in the natural group (Figure 4.4A) and this was the stage observed in all the stimulation groups, apart from the PMSG group, which had already begun to undergo the first cell division at the time of embryo collection (Figure 4.4F). The PMSG group therefore reached the blastocyst stage either on Day 4 or 5, whereas all the other experimental groups reached this milestone on Day 5 (Figure 4.4F).

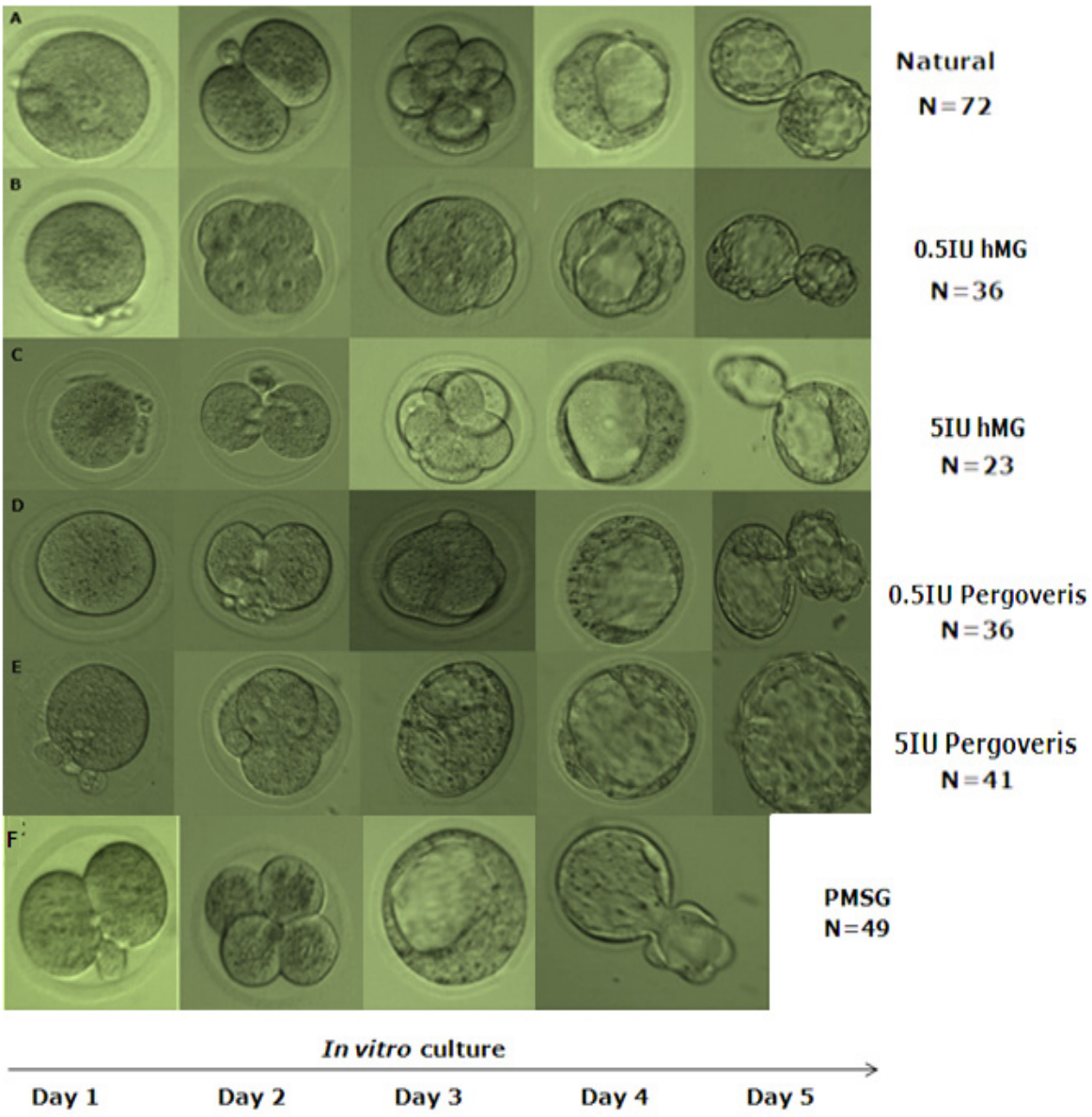

Figure 4.4: Daily monitoring of embryo development throughout *in vitro* culture.

On Day 2 of development the naturally mated group had undergone the first cell division. This initial cleavage in the 5IU hMG and 0.5IU Pergoveris stimulation groups showed some fragmentation. Groups treated with 0.5IU hMG, 5IU Pergoveris and PMSG on Day 2 had already reached the 4 cell stage. This accelerated development was still evident on Day 3 where the 0.5IU hMG and 5IU Pergoveris groups had already begun the process of compaction whereas the natural group was at the 8 cell pre-compaction phase. The 0.5IU Pergoveris group had also started to undergo

compaction. The PMSG group already had a clearly evident blastocoel cavity on Day 3 and some groups on Day 4 had started to hatch out of the zona pellucida. On Day 4 embryos from all experimental groups, apart from treatment with PMSG, looked morphologically similar, with a clearly evident blastocoel cavity and all embryos reaching the blastocyst stage on Day 5.

4.3.2.3 Developmental stage at media change

The developmental stage which embryos had reached on Day 3 at the time of media change, are summarised in Figure 4.5. The proportion of embryos from naturally mated mice at the morula stage of development was comparable to treatment with 0.5IU Pergoveris ($P>0.05$). In contrast stimulation with PMSG, 0.5IU hMG and 5IU Pergoveris as previously indicated, resulted in significantly more embryos at the morula stage ($P<0.001$). Treatment with 5IU hMG, on the other hand resulted in a decline in the number of embryos reaching compaction ($P<0.05$) with a significant proportion of these embryos remaining at the 2-4 cell stage of development compared to natural controls ($P<0.001$). Treatment with 0.5IU Pergoveris also resulted in an increase in the number of embryos arresting at this early developmental stage ($P<0.001$). The PMSG, 0.5IU hMG and 5IU Pergoveris groups were comparable to embryos from naturally cycling animals with regard to the proportion of embryos not progressing from the 4 cell stage ($P>0.05$). The majority of naturally derived embryos on Day 3, at the time of media change, were at the 6-8 cell pre-compaction stage of embryo development. This was significantly different in all the ovarian stimulation groups ($P<0.001$), either because embryos in these groups were more advanced, for example the PMSG, 0.5IU hMG and 5IU Pergoveris groups, or delayed, as was the case for treatment with 5IU hMG and 0.5IU Pergoveris.

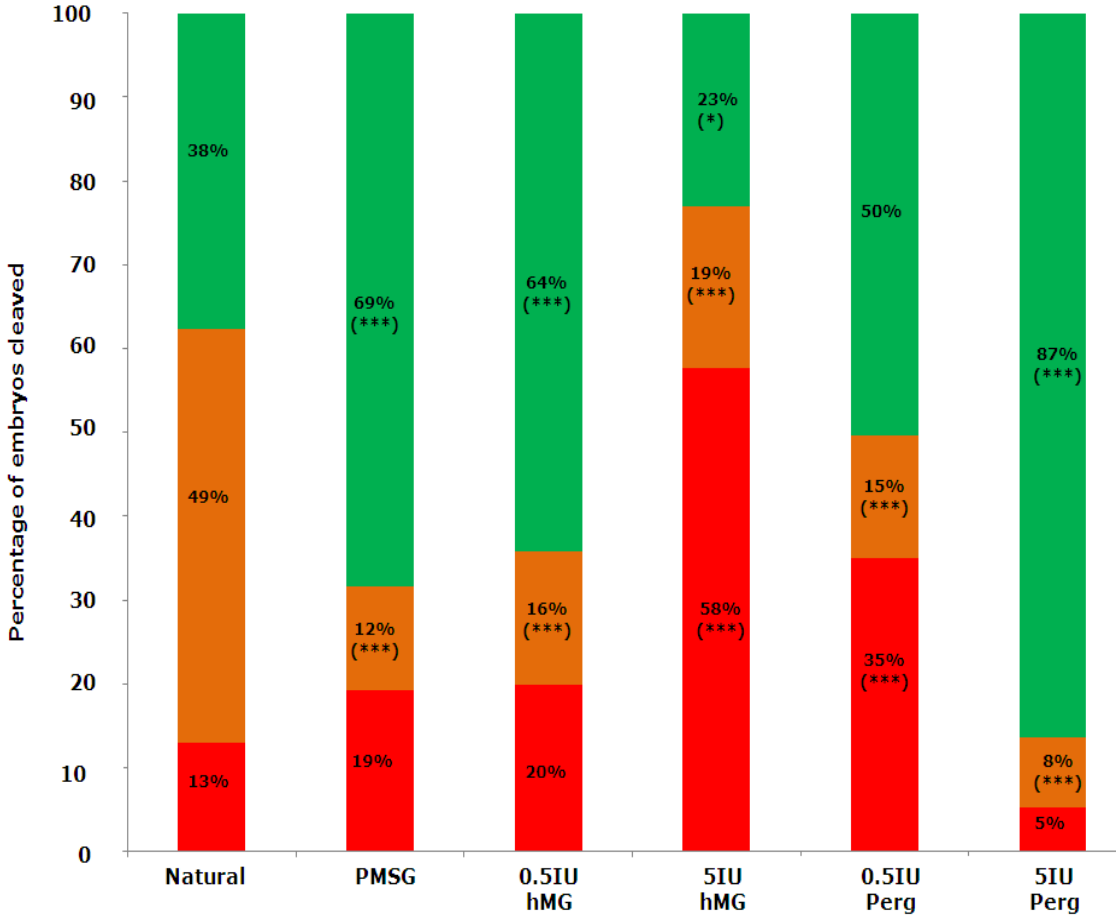

Figure 4.5: Proportion of cleaved embryos on day 3 at ≤4 cells (red), 6-8 cells (orange) and the morula (green) morphological stage of development for the Natural, PMSG, 0.5IU hMG, 5IU hMG, 0.5IU Pergoveris, 5IU Pergoveris, 0.5IU rFSH, 2.5IU rFSH, 5IU rFSH. (statistical significant of comparison to naturally mated mice at same developmental stage; *** P<0.001, *P<0.05 as assessed by chi square test for associations).

4.3.2.4 Timing of embryo development by Embryoscope

Naturally derived embryos cleaved to the 2 cell stage after 37±2.7 (Median ± inter-quartile range) hours (Figure 4.6, Representative videos are also available on the CD Rom accompanying this thesis). The next two rounds of cell divisions occurred at 60±5.3 and 71±5.4 hours. Compaction of the embryo occurred at 82±4.5 hours with cavitation first observed at 100±4.3 hours. Embryos became fully expanded blastocysts at 117±10.2 and eventually began to hatch at 120 hours.

The ovarian stimulation groups had significantly different timings of development compared to naturally derived embryos (Figures 4.6-4.9). As previously indicated, treatment with PMSG resulted in accelerated development, with embryos undergoing the first and second cell divisions at 32±1 and 56±4.8 hours post hCG injection ($P<0.001$) and reaching the 6-8 cell stage at a comparable time as control embryos (72 hours, $P>0.05$, Figure 4.6). However in comparison to other treatment groups and the negative control, embryos from PMSG treated mice underwent compaction at the 4 cell stage, instead of after reaching 6-8 cells. Only 2 embryos out of the 12 analysed progressed to the 6-8 cell stage prior to morula formations in the PMSG treatment group ($P<0.01$). Timing of cavitation was comparable with control at 105±16.7 hours ($P>0.05$), however further development into a full blastocyst was significantly delayed in the PMSG group (132 hours, $P<0.05$) although blastocysts hatching timing was comparable (139 hours, $P>0.05$).

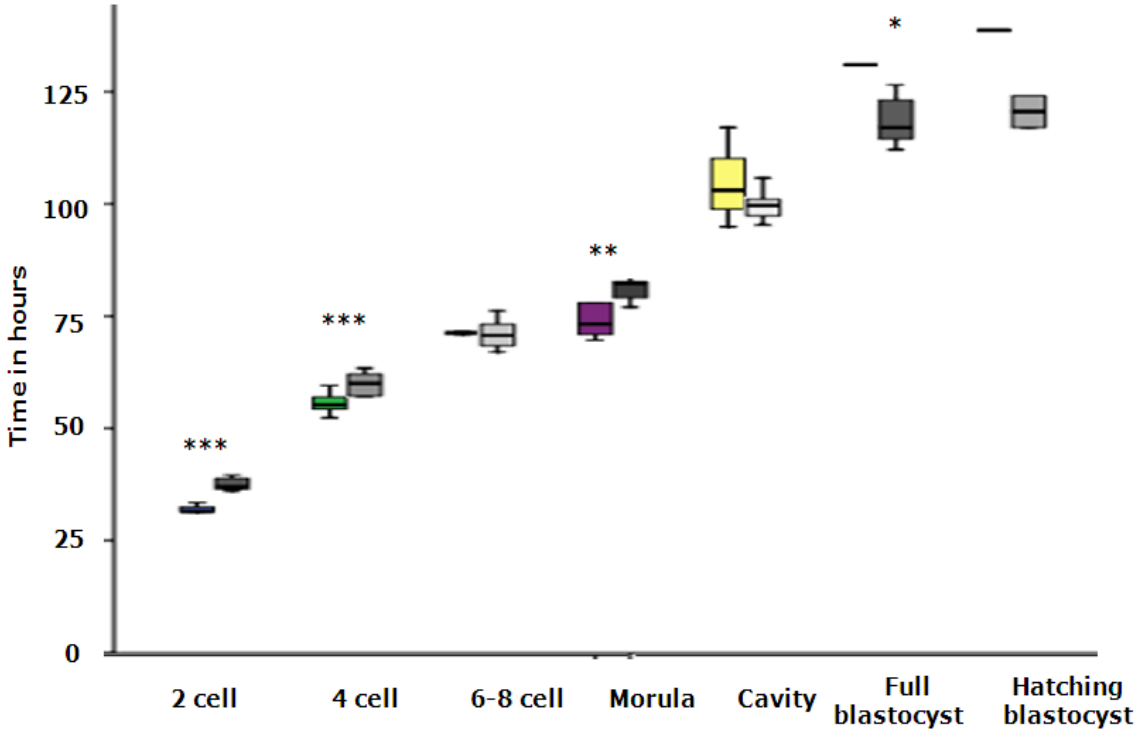

Figure 4.6: Median time for embryos following treatment with PMSG (colour box plot) to reach developmental milestones compared to embryos derived from naturally mated controls (Greyscale box plot, n=12 per group). Error bars show inter-quartile range. (*** $P<0.001$, ** $P<0.01$ and * $P<0.05$ compared to control as analysed by Mann-Whitney U test).

Daily analysis of embryos from mice stimulated with 5IU hMG indicated development occurred at a comparable rate to embryos derived from naturally cycling mice; however the embryoscope data indicated that this treatment also accelerated the speed of development up until compaction (Figure 4.7). Thus, the first cell division occurred significantly earlier at 32±4.8 hours post hCG ($P<0.001$). Subsequent development to the 4 and 6-8 cell stage was also advanced, occurring at 53±6.7 and 66±6.3 hours respectively ($P<0.05$). Despite compaction also occurring earlier (74±6.1 hours, $P<0.05$), morphological milestones after this developmental stage were similar to the un-stimulated group ($P>0.05$).

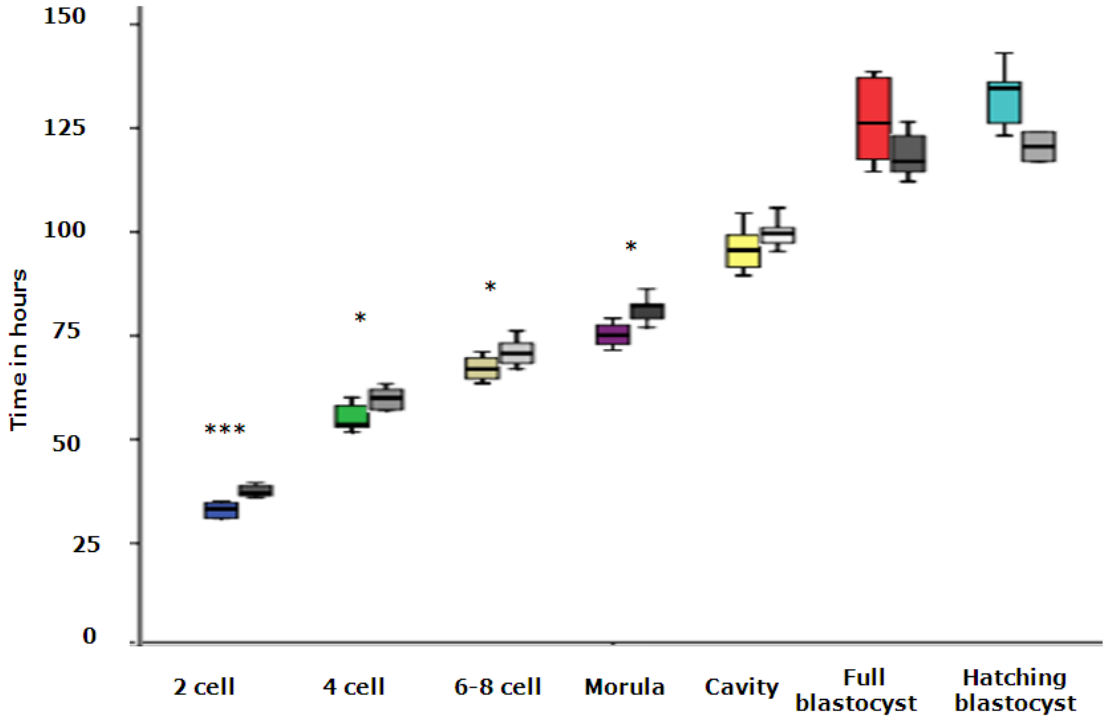

Figure 4.7: Median time for embryos following treatment with 5IU hMG (colour box plot) to reach developmental milestones compared to embryo derived from naturally mated controls (Greyscale box plot, n=12 per group). Error bars show inter-quartile range. (*** $P<0.001$ and * $P<0.05$ compared to control as analysed by Mann-Whitney U test).

Development of embryos following treatment with 0.5IU Pergoveris was also significantly accelerated until the time of cavity formation (Figure

4.8). Cleavage in this treatment group to the 2, 4 and 6-8 cell stage occurred significantly earlier compared to natural controls (P<0.01). Upon reaching the 6-8 cell stage, compaction into the morula occurred almost instantaneously at 59±19.1 hours, which was slightly less time taken to reach the 6-8 cell stage, and significantly earlier than unstimulated controls (P<0.001). This consequently meant cavitation also occurred at an earlier time (94±5.9, P<0.01). However, as noted with daily monitoring of embryo development, the embryoscope data confirmed this group reached the full and hatching blastocyst stage at analogous times to the natural group at 118±7.3 and 121±12.5 hours (P>0.05).

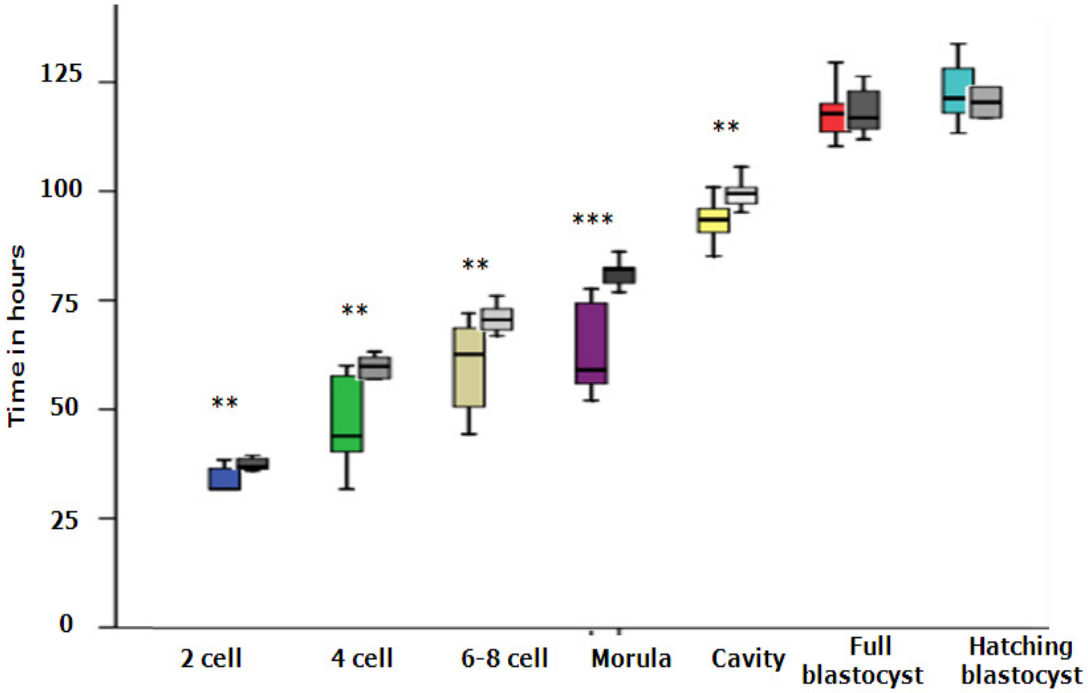

Figure 4.8: Median time embryos following treatment with 0.5IU Pergoveris (colour box plot) to reach developmental milestones compared to embryo derived from naturally mated controls (Greyscale box plot, n=12 per group). Error bars show inter-quartile range. (*** P<0.001 and ** P<0.01 compared to control as analysed by Mann-Whitney U test).

Daily monitoring of development of embryos derived from the 5IU Pergoveris treatment indicated that this group also had accelerated development to the hatching blastocyst stage. This finding was further supported by the embryoscope data (Figure 4.9). Initial cleavage occurred

significantly earlier compared to controls and all other treatment groups at only 30±2.4 hours post hCG (P<0.001). Further cleavage to the 4 and 6-8 cell stages were subsequently earlier at 54±4.8 and 66±4.2 hours (P<0.001 and P<0.01 respectively). Unlike the other ovarian stimulation groups this pattern of accelerated embryo development continued until the full blastocyst stage with compaction, cavity and full blastocoel formation all occurring earlier (P<0.001). Although hatching blastocysts from this group were observed earlier at 110±6.9 hours post hCG, this trend was not statistically significant (P>0.05).

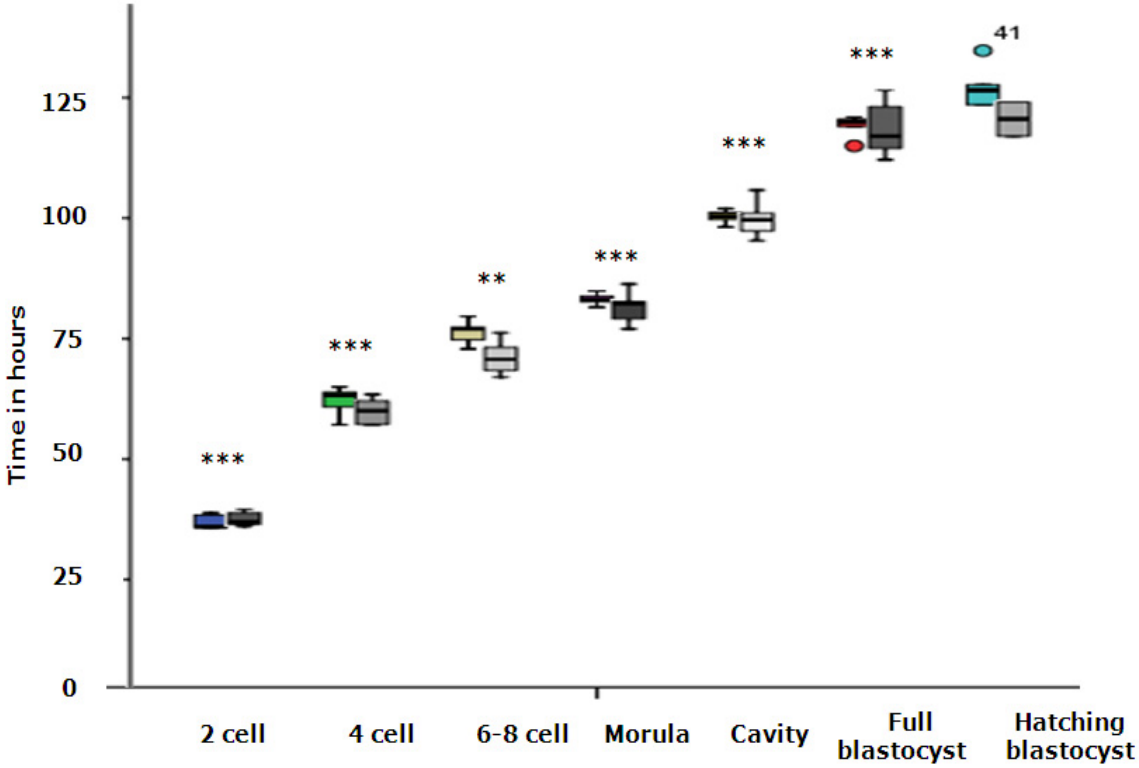

Figure 4.9: Median time embryos following treatment with 5IU Pergoveris (colour box plot) to reach developmental milestones compared to embryo derived from naturally mated controls (Greyscale box plot, n=12 per group). Error bars show inter-quartile range. (*** P<0.001 and ** P<0.01 compared to control as analysed by Mann-Whitney U test).

4.3.2.5 DAPI staining of arrested embryos

Embryos that had arrested development prior to media change exhibited a number of different abnormalities, as illustrated in Figure 4.10. Unequal distribution of the cytoplasm and genetic material was evident, as was

failure to separate the metaphase plate. This arrested development appeared to only be evident in the ovarian stimulation groups and was not present in any of the arrested embryos from the un-stimulated controls.

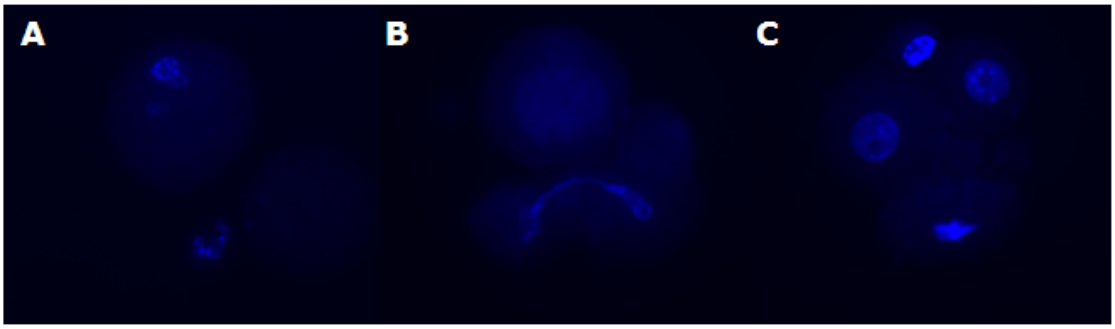

Figure 4.10: DAPI stained embryos arrested at the **(A)** 2 cell, **(B)** 3 cell and **(C)** 4 cell stage.

4.3.2.6 Blastocyst development

Only ovarian stimulation with 0.5IU hMG and 5IU Pergoveris resulted in a significant increase in the number of blastocysts obtained relative to the un-stimulated control group (Figure 4.11, $P<0.001$). The positive control PMSG stimulation regime did not result in an increase in the number of embryos per mouse developing to the blastocyst stage ($P>0.05$) when compared to untreated controls. The 5IU hMG and 0.5IU Pergoveris treatment groups actually resulted in less embryos developing to blastocyst compared to controls but this trend was not statistically significant ($P>0.05$).

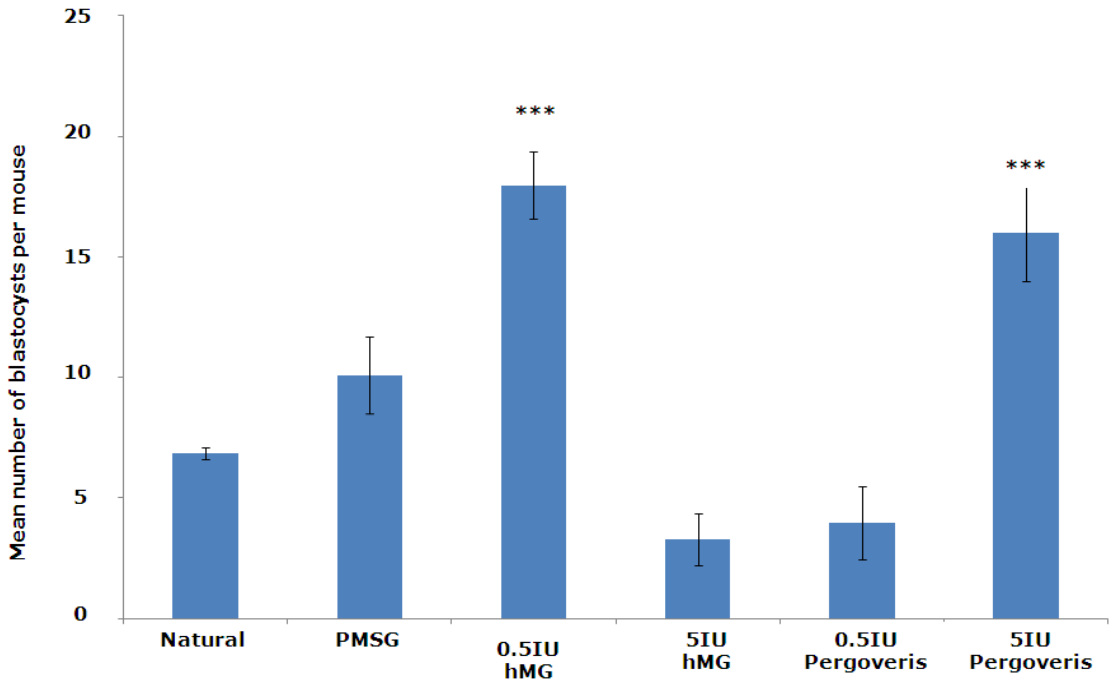

Figure 4.11: Mean ± SEM number of embryos developing to the blastocyst stage per mouse for Natural, PMSG, 0.5IU hMG, 5IU hMG, 0.5IU Pergoveris and 5IU Pergoveris. (*** P<0.001 compared to control as assessed by ANOVA).

4.3.2.7 Blastocyst grade

The proportion of blastocysts classified as early, fully expanded, hatching and hatched (Figure 4.12) were comparable between the ovarian stimulation regimes and the natural control (P>0.05), except for treatment with 5IU Pergoveris. This treatment group had significantly more hatching blastocysts (P<0.001) and a reduced proportion of early blastocysts (P<0.05) when compared to untreated controls.

Eighty eight percent of blastocysts derived from naturally cycling animals were graded 3BB or better according to Gardner and Schoolcrafts grading system, with the majority being classified as 5AA-5BB (Figure 4.13). The quality of embryos derived from the PMSG treatment group was comparable to controls with most graded as 5AA- 5CC. However, this stimulation group also had some poorer 3CC blastocysts; in agreement with the embryoscope data which indicated that this treatment resulted in

a delay in development of fully expanded blastocysts (see 4.3.2.4). The 0.5IU hMG treatment resulted in 81% of blastocysts being graded as over 3BB and although most were graded 5AA- 4BB, below this classification the quality varied across the lower grade boundaries.

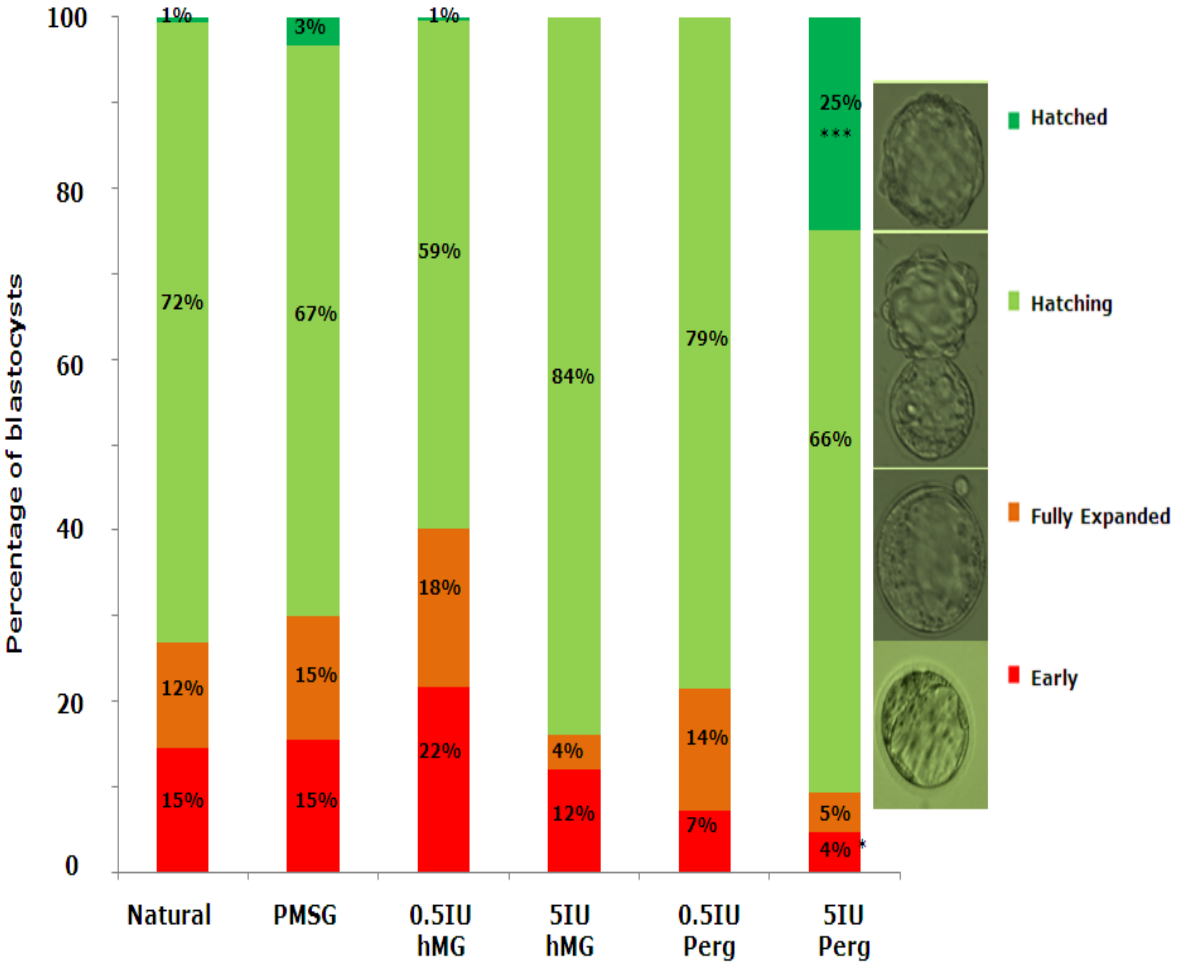

Figure 4.12: Proportion of blastocysts in each morphological classification based on treatment (Natural (n=72), PMSG (n=49), 0.5IU hMG (n=36), 5IU hMG (n=23), 0.5IU Pergoveris (n=36), 5IU Pergoveris (n=41)). (*** P<0.001, * P<0.05 compared to control proportions as analysed by chi square test for associations).

Treatment with 5IU hMG resulted in 88% good quality blastocysts being graded as 3BB or higher, with the majority being graded as 5AA-5CC. However, this group also had a noticeable proportion of low quality 1CC grades, although this trend was not statistically significant (P>0.05). Both the stimulation regimes utilising Pergoveris resulted in higher embryo

grades than unstimulated controls with 93 and 97% graded 3BB or better for the 0.5IU and 5IU doses respectively. However, only the 5IU Pergoveris treatment resulted in a statistically significant (P<0.05) increase in embryo quality with a predominant representation in the grade 6 category (P<0.001).

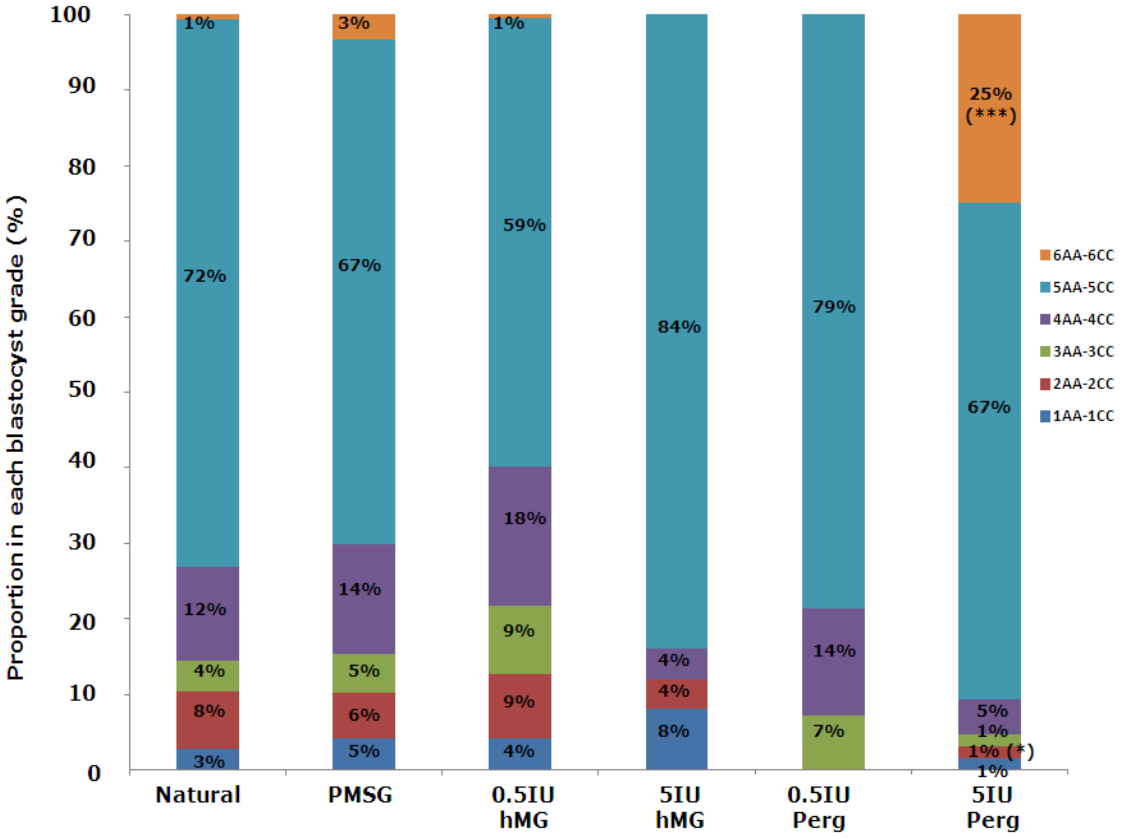

Figure 4.13: Proportion of blastocysts in each grade based on treatment. (*** P<0.001 and * P<0.05 compared to natural control as assessed by chi square test for associations).

4.3.2.8 Blastocyst triple staining

The unstimulated control group had a median total cell number (TCN) of 81 per hatched or hatching blastocyst (Table 4.1). Treatment with PMSG resulted in a 20% decrease in TCN compared to controls (P<0.05). In contrast blastocysts from the 0.5IU and 5IU Pergoveris treatment groups had a 38% and 58% increase in cell number respectively (P<0.001). Blastocysts from the remaining stimulation groups contained comparable total cell numbers as untreated controls apart from the 0.5IU hMG treated

group which also had a significant increase in TCN (P<0.01). The proportion of cells allocated to the ICM or TE was similar within all treatments groups (Table 4.1) although embryos from the PMSG group tended to distribute more cells to the trophectoderm than the ICM (Table 4.1; P=0.059).

The proportion of apoptotic cells within the blastocysts from across all treatment groups tended to be relatively low with blastocysts from nonstimulated controls, 0.5IU hMG, and 0.5IU Pergoveris treatment groups having around 5% apoptotic cells. The proportion of apoptotic cells was almost doubled when ovarian stimulation was performed with PMSG or 5IU hMG (P<0.05). In contrast the 5IU Pergoveris treatment tended to reduced the proportion of apoptotic cells and this difference approached statistical significance (P=0.068). Figure 4.14 displays representative blastocysts for all the treatment groups from which the cell counts were obtained.

Table 4.2: Blastocyst triple staining counts for all treatment groups (Natural (n=72), PMSG (n=49), 0.5IU hMG (n=36), 5IU hMG (n=23), 0.5IU Pergoveris (n=36), 5IU Pergoveris (n=41)). (* P=<0.05, *** P=<0.001 compared to control as assessed by chi square and Mann-Whitney U tests).

Treatment	Total Cell numbers (TCN)	ICM proportion (%)	TE proportion (%)	Proportion of apoptotic cells (%)
Natural	81(47)	46	54	5
PMSG	70(57)*	39	61	9*
0.5IU hMG	110(108)**	45	55	5
5IU hMG	93(44)	39	61	9*
0.5IU Pergoveris	112(37)***	40	60	5
5IU Pergoveris	128(71)***	41	59	3

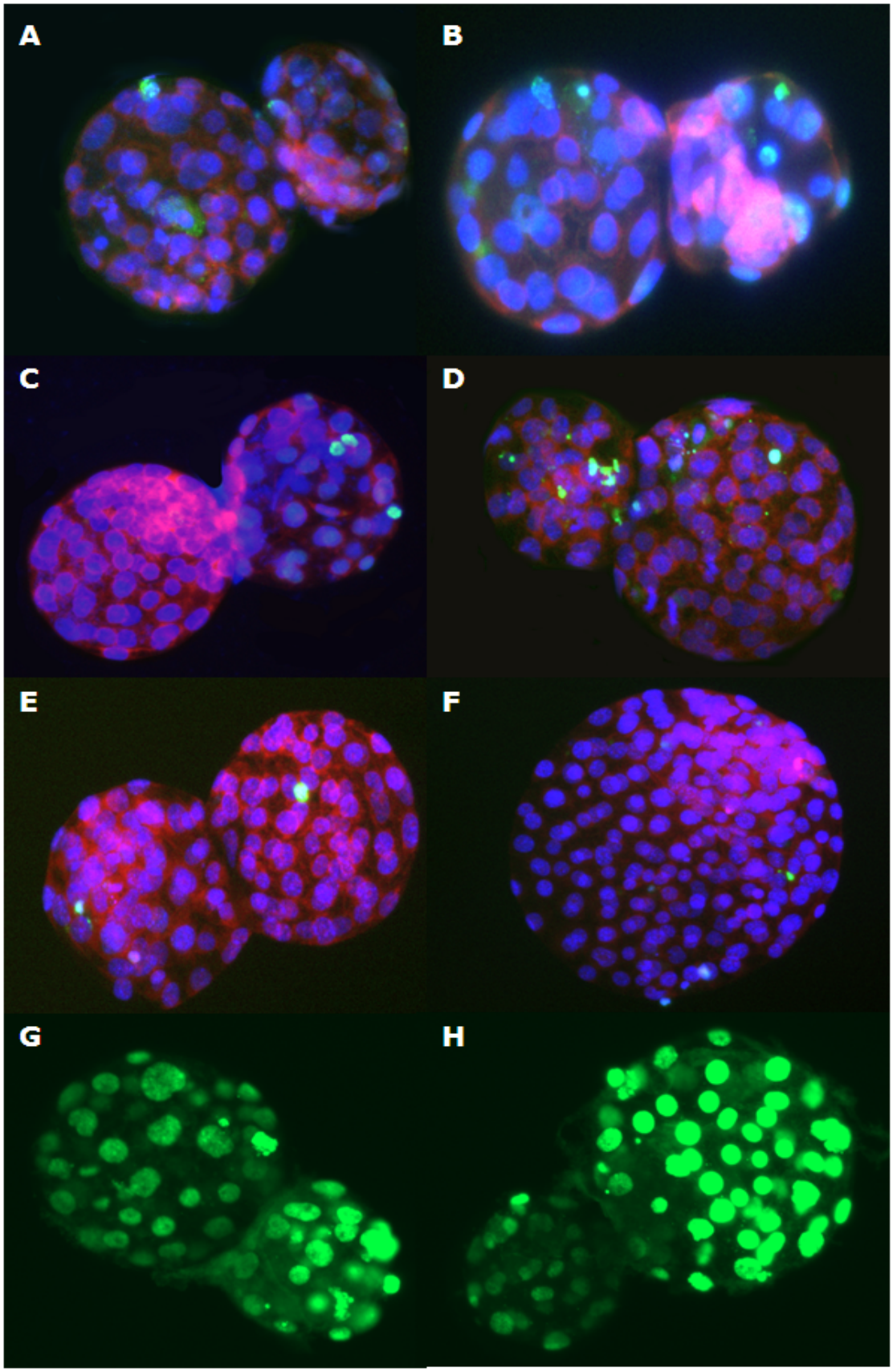

Figure 4.14: Representative DAPI, PI and Tunel triple stained blastocysts for **(A)** Natural, **(B)** PMSG, **(C)** 0.5IU hMG, **(D)** 5IU hMG, **(E)** 0.5IU Pergoveris and **(F)** 5IU Pergoveris groups and **(G-H)** Positive tunel controls

4.4 DISCUSSION

In this study it was hypothesised that the use of human gonadotrophin preparations for ovarian stimulation in mice would not be detrimental to embryo development as has been reported for PMSG. The results obtained suggest that whilst superovulation with PMSG is detrimental to *in vitro* development to the blastocyst stage, contrary to the initial hypothesis, ovarian stimulation with human preparations also had both negative and positive effects on both embryo development and embryo quality relative to unstimulated controls. This study provides further evidence of the effects of ovarian stimulation with different gonadotrophin preparations on embryo development, highlighting the complexity of effects resulting from the use of different doses of gonadotrophin preparations which vary in terms of their biological clearance rate, FSH isoforms and level of LH-bioactivity, such as hCG in hMG.

4.4.1 PMSG effect on embryo yield and development

Ovarian stimulation with PMSG is the standard protocol used to induce multiple follicular development in the mouse (Nagy *et al.*, 2003). The mean zygote yield of 25 observed in this study for COS with PMSG is consistent with those reported in the literature ranging from 17-33 across the various mouse strains (Martín-Coello *et al.*, 2008; Edgar *et al.*, 1987; Muñoz *et al.*, 1995; Kanter *et al.*, 2004; Brooke *et al.*, 2007). In agreement with previous studies treatment with PMSG resulted in reduced *in vitro* development, with significant developmental arrest and a blastocyst yield of approximately 50%, which is comparable to the published rates of 50-54% when the whole ovulated group per mouse is taken into account (Edgar *et al.*, 1987; Muñoz *et al.*, 1994). The proportion of hatching blastocysts on Day 5 in the PMSG group is comparable to previously reported rates in the literature of 64.9% (Ghaemi *et al.*, 2008). Blastocysts obtained after PMSG treatment, despite having high rates of developmental arrest, had an accelerated rate of development reaching morphological checkpoints in pre-implantation development faster than untreated controls.

It is possible that this apparent increase in development is an artefact due to the LH activity of PMSG resulting in induction of ovulation and thus oocyte fertilisation earlier than naturally ovulated counterparts. However, this appears unlikely as the COCs were recovered from the same region of the oviduct in both PMSG treatment and controls groups. Further, flushing of the uterine horns of PMSG stimulated mice has not been shown to yield any further zygotes (Ozgunen *et al.*, 2001), suggesting that premature ovulation is not occurring in response to stimulation with PMSG. Ninety percent of the embryos from the PMSG treated group underwent compaction prematurely at the 4 cell stage whereas embryos from nonstimulated and other ovarian stimulation groups underwent an additional cleavage step to the 6-8 cell stage before morula formation, therefore potentially explaining the increased developmental speed of embryos from PMSG treated animals. The total cell numbers of un-stimulated and PMSG blastocysts calculated in our study are analogous with previous reports (Ghaemi *et al.*, 2008; Tarín *et al.*, 2002) with blastocysts from PMSG treated animals having significantly fewer cells. This decrease in cell number may potentially be a consequence of the increased developmental speed and the observed loss of a cleavage step during the *in vitro* development of embryos from PMSG treated groups. The negative effects of PMSG may be partly due to the long acting LH bioactivity in the preparation. In a previous study increased doses of PMSG not only halved the number of oocytes, but reduced the proportion of embryos cleaving to the two cell stage and developing to blastocyst significantly to only 38% and a mere 7% respectively (Edgar *et al.*, 1987). Although not assessed in our study, analogous results were obtained with human gonadotrophin preparations containing hCG as discussed in section 4.4.5.

4.4.2 Ovarian stimulation with human gonadotrophins

There have been few studies in the literature investigating human gonadotrophins in mouse ovarian stimulation. Human menopausal gonadotrophin (hMG) like PMSG has approximately equal amounts of FSH and LH bio-activity (Lunenfeld, 2004) although the relative half-lives of

these heterologous gonadotrophins in the mouse are unknown. Similarly, as the bio-assay used to determine the biological activity of both PMSG and hMG differ (Macklon *et al.*, 2006), it is not possible to directly compare the doses utilised in this study for ovarian stimulation. Previous studies assessing hMG for mouse superovulation used earlier formulations of the preparation where the LH activity came from urinary LH as opposed to hCG in the preparation used in our study. These studies have reported only 15 zygotes obtained after a single dose of 10IU (Edirisinghe *et al.*, 1986) and a mean of 34 when 2 repeated doses of 1IU 12 hours apart were given (Brooke *et al.*, 2007). Although previous reports have concluded a comparable number of embryos are achieved by hMG and PMSG, no investigations into embryo development was undertaken, as the outcome of previous experiments was increasing the yield of oocytes for transgenic applications (Brooke *et al.*, 2007). Our results suggest repeated injections of 0.5IU hMG increases the number of blastocysts compared to both the negative natural control and positive PMSG control and the quality of the resulting 0.5IU hMG blastocysts were comparable to naturally derived blastocysts in terms of grade and fluorescent analysis outcomes. 5IU Pergoveris also showed more favourable results in terms of blastocyst rate and quality whereas stimulation with pure rFSH alone did not result in any blastocysts being produced. These differences in embryo quality and number following ovarian stimulation can be largely attributed to differences in the LH activity, FSH activity and half life of the gonadotrophic preparations tested.

4.4.3 rFSH alone

Treatment with rFSH alone in our study did not yield any blastocysts, however, these results are in direct conflict compared to the only previous study to our knowledge assessing rFSH in the mouse model where comparable total doses of rFSH, given 24 hours apart as opposed to every 12 hours in our study, resulted in a 72% blastocyst rate in mature mice of the same strain (Edwards *et al.*, 2005). In our study we utilised immature mice to overcome the need for down regulation, without which premature ovulation may occur in mature mice that may potentially

provide an explanation for the discrepancy between our results and the literature. However after repeating the 2.5IU rFSH protocol with mature mice at 8 weeks of age blastocyst formation was still not evident.[7] The reason for the discrepancy in results between our study and the previous published is unclear. This research group used the same CBA X C57Bl/6 F1 female strain however several sub-strains of CBA X C57Bl/6 F1 do exist and genetic drift as a consequence of the isolation of breeding colonies over several years may be a factor (Zurita *et al.*, 2011). The use of different culture medium and *in vitro* culture incubator conditions may also explain the differences in results. The authors did conclude that embryo development after treatment with rFSH was poor and resulting blastocysts had reduced cell numbers (Edwards *et al.*, 2005).

Our study also showed that regimes with rFSH alone did not yield developmentally competent embryos. This is a surprising result as this standard rFSH stimulation regime has been found to be highly effective in humans (van Wely *et al.*, 2011). Ovarian stimulation regimes composed of high doses of rFSH only work by maintaining FSH above threshold concentrations for an extended period so more ovulatory follicles recruited from the gonadotrophin-responsive group are maintained as FSH-dependent until the ovulatory trigger is applied (Campbell *et al.*, 1999; Brown, 1978; Schipper *et al.*, 1998). Mice have a higher metabolic rate compared to humans and based on the evidence that rFSH contains a high proportion of less acidic iso-forms (Lispi *et al.*, 2006) which show greater *in vitro* bioactivity but have a faster *in vivo* clearance rate (Vitt *et al.*, 1998), suggests this preparation may not be sufficient to maintain FSH levels above the threshold. Due to home office license restrictions, the frequency of the injections could not be increased to assess this theory.

It could be suggested that poor embryo quality associated with rFSH alone is an artefact of the mouse model and reflects fluctuations of the FSH

[7] See Appendix 5 for data.

level above and below the threshold, due to the preparations short half life. The observation that identical FSH concentrations following treatment with Pergoveris, which has rLH in addition, does yield developmental competent embryos indicates that the presence of LH, whether endogenous in not fully down regulated patients or exogenous from preparations such as Pergoveris or hMG can act to support the follicles when FSH levels fall below the threshold. During the normal follicular phase ovulatory follicles transfer their gonadotrophin dependence from FSH to LH as part of the normal follicle selection process (Huirne *et al.*, 2004; Campbell *et al.*, 1999), explaining why preparations with LH produce viable embryos as discussed further in section 4.4.4.

4.4.4 Combined rFSH and rLH (Pergoveris)

The production of good quality embryos after treatment with Pergoveris can be attributed to the ability of the ovulatory follicles to transfer dependence from FSH to LH so gonadotrophic support is adequate until the ovulatory trigger is applied (Campbell *et al.*, 1999). Of the two doses tested only the higher dose resulted in increased embryo yield and improved proportions of good quality blastocysts graded 3BB or higher according to Gardner and Schoolcrafts system. In particular, there were more hatching blastocysts at higher doses of Pergoveris and a trend for reduced apoptosis rates which have all been demonstrated as indicators of improved quality which translate into better implantation and ongoing pregnancy rates (Alper *et al.*, 2001; Teranishi *et al.*, 2009; Brinsko *et al.*, 1994; Fong and Bongso, 1999).

The lack of response using the lower dose of Pergoveris and the subsequent poor embryo development can probably be attributed to the short half life of rFSH as discussed in section 4.4.3. The timing of developmental arrest at the two cell stage is coincidental with mouse embryonic genome activation, indicating poor oocyte quality as a result of the ovarian stimulation regime may be responsible (Gardner and Lane, 2005; Wassarman and Kinloch, 1992). Fluorescent analysis of these

arrested embryos showed unequal division of genetic material which at such early stages of development would potentially depend on the oocytes potential to modulate these events (Pesty *et al.*, 2007). Higher concentrations of rFSH appear to be required in order to produce fully competent oocytes capable of continuing embryonic development after ZGA.

4.4.5 Urinary FSH with long half life LH activity

In stark contrast to Pergoveris, the lower dose of hMG resulted in an increase in blastocyst yield whereas the higher dose was detrimental. This difference in response can most likely again be attributed to the longer half lives of the urinary FSH and hCG contained within hMG. Although it is also clear that hMG contains more LH activity (1:1) than Pergoveris (2:1). Thus lower doses of hMG could have supplied sufficient gonadotrophic support to maintain the ovulatory follicles (as per high doses of Pergoveris) due to the longer *in vivo* half life of these preparations (West *et al.*, 2002) as a result of the mostly acidic iso-forms in the urinary FSH (Lispi *et al.*, 2006). Higher doses of hMG, in contrast, caused a reduction in the number of oocytes ovulated and resulted in poor embryo development and blastocyst formation with higher levels of apoptosis. This could potentially be due to the supply of un-physiologically high levels of LH stimulation due to the modified extension of the β subunit of hCG extending its half life (Huirne *et al.*, 2004) in a manner analogous to PMSG.

Hillier suggested that each follicle has a LH ceiling threshold, above which normal maturation of the follicle ceases, proposed to be associated with high levels of cAMP stimulation which is known to be major regulator oocyte maturation (Hillier, 1994), potentially explaining our alternative results for hCG and rLH containing preparations. The mechanistic effect is unclear but previous studies have shown that high concentrations of LH and hCG may lead to down regulation of the LH receptor on granulosa cells in human (Jeppesen *et al.*, 2012) and rats (Rao *et al.*, 1977; Peng *et*

al., 1991), confirmed by real time PCR, *in situ* hybridisation and northern blots. *In vitro* studies suggested long term exposure to LH can result in a conformational changes of the receptor that reduces the rate of phosphorylation on subsequent ligand binding (Minegishi *et al.*, 1989), whereas others have suggested loss of receptors through internalisation and degradation may be involved or by reduction in LH receptor synthesis (Schwall and Erickson, 1984). Regardless of the putative mechanism, hCG has consistently been shown to reduce LH receptors by approximately 80% within 24 hours following a large bolus injection (Jeppesen *et al.*, 2012; Rao *et al.*, 1977), after which re-exposure results in a decreased response.

Reduced LH receptors may reduce the number of follicles responsive to LH during the later stages of folliculogenesis which was shown to be beneficial, as per treatment with Pergoveris and in addition provides a potential explanation as to why ovulation rates were reduced at higher doses of hMG. Therefore in preparations containing hCG, the LH bioactivity resulting from increasing the dose may lead to LH levels above a hypothesised ceiling level causing detrimental embryo development, whereas the low half life of recombinant LH results in the Pergoveris preparation being within the theorised LH window, providing beneficial effects. It is unclear how these preparations affect follicle development, but the manifestation of the preparations influence on subsequent embryo development is clear.

4.4.6 Conclusion

The results of this study show clearly that the quality of embryos obtained following ovarian stimulation of mice with human gonadotrophin preparations is critically dependent on the dose, half life and LH content of the preparations used. Although we were unable to obtain blastocysts from regimes utilising rFSH alone, the results strongly indicate that good quality embryos can be obtained from low doses of urinary preparations containing FSH and LH activity with longer halve lives or higher doses of

recombinant preparations containing FSH and LH activity with much shorter half lives. Further, these studies have confirmed and extended the observation that ovarian stimulation with high doses of gonadotrophins with extended LH activity (PMSG, hMG) results in embryos of poor quality, although the exact mechanisms resulting in this response remain unclear. Future studies described in the following chapters of this thesis will examine the molecular and developmental consequences of the differences in embryo quality induced by these different stimulation regimes.

CHAPTER 5: OVARIAN STIMULATION AND GENE EXPRESSION IN THE EMBRYO

5.1 INTRODUCTION

In recent years, our knowledge of pre-implantation processes and the role genetics plays in fine-tuning these events has vastly improved. Focus is now being directed at assessing the relationship between ART procedures and genetic alterations. One of the main areas of interest has been imprinted gene expression. Whilst the absolute risk of developing an imprinted disorder is low, children born through ART do appear to be more susceptible than those in the general population (Maher, 2005). There have been numerous reports correlating genomic imprinting errors and disorders with ART, in animal models (Market-Velker *et al.*, 2010; Menezo *et al.*, 2010; Zhang *et al.*, 2010; Mundim *et al.*, 2009) and human subjects (Strawn *et al.*, 2010; Katari *et al.*, 2009; Lawrence and Moley, 2008; Sato *et al.*, 2007). Finding the aspect of the ART procedure responsible for these risks has remained elusive. Due to the low incidence of imprinting disorders after ART, it is extremely difficult to explore cause-effect relationships from clinical data. Therefore, precisely defined *in vitro* models are powerful tools for identifying mechanisms.

Methylation disturbances have been reported throughout the oocyte and embryonic genome after ovarian stimulation (Shi and Haaf, 2002; Sato *et al.*, 2007) suggesting that superovulation may be the source of imprinting defects. Epigenetic marks are gained in an asynchronous manner throughout folliculogenesis in correlation with oocyte diameter, with most of the methylation occurring in the diplotene stage of oocyte meiosis in mice and humans (Lucifero *et al.*, 2002). The unphysiological hormonal milieu due to exogenous gonadotrophin administration during this phase may perturb imprints. It has been demonstrated previously that methylation alterations are exacerbated by ovarian stimulation, in a dose dependent manner (Market-Velker *et al.*, 2010).

There are 96 known conserved murine and human genes that exhibit parent-of-origin specific methylation patterns (Wood and Oakey, 2006). The *H19* and *IGF2* gene clusters have received the most attention as their expression is hypersensitive to disruptions during gametogenesis. Hence, they are frequently used as a quasi-sensor for epigenetic perturbations (Nan *et al.*, 1998; Doherty *et al.*, 2000). *IGF2* is a potent foetal mitogen that is regulated by *H19* through a shared differentially methylated region (De Rycke *et al.*, 2002; Leighton *et al.*, 1995). They exert their effects on the placenta regulating nutrient transfer and coincidently foetal growth and development (De Rycke *et al.*, 2002).

In addition to imprinted gene expression, in recent years the relationship between vascularity of the ovary and follicle with ART outcomes has also been investigated. The renin-angiotensin system (RAS) has been shown to play a pivitol role in decidualisation, implantation and placentation through the regulation of blood flow, oestradiol secretion and prostaglandin synthesis (Nielsen *et al.*, 2000). Disruption of the RAS has been implicated in cases of pre-eclampsia and IUGR (Li *et al.*, 1998). Binding of angiotensin to the AT_1 receptor (ATR1), predominately found on EVT, promotes growth processes that are required throughout pregnancy. AT_2 receptors (ATR2) on the other hand, inhibit cell proliferation and leads to cell apoptosis (Nielsen *et al.*, 2000). Changes in the relative expression of *ATR1* and *ATR2* have been proposed to determine whether angiotensin promotes growth, apoptosis or differentiation. The pro-angiogenic factor *VEGF*, is one of the final products of the RAS pathway (Agrawal *et al.*, 2002). Oestrogen has recently been shown to stimulate *VEGF* expression in the ovary (Ha *et al.*, 2010). Disruption of the RAS due to exogenous gonadotrophin administration may cause imbalances in the uterus inhibiting implantation. No previous work has focused on how ovarian stimulation may alter this complex angiogenic system.

The objective of this experiment was to examine the effect of common human gonadotrophin preparations (hMG and Pergoveris) on the expression of genes regulating imprinting and angiogenesis. The

endpoints measured were *H19, IGF2* and *IGF2r* gene expression for epigenetic disturbances and *VEGFA, ATR1* and *ATR2* expression levels as vascularity indexes. Based on previous oocyte and embryo development analyses (Chapter 3 and 4) it was hypothesised that the use of human gonadotrophin preparations for ovarian stimulation in mice would not be as disruptive to imprinted gene expression as preparations containing long acting LH (PMSG/hMG).

5.2 METHODS OVERVIEW

Mice were randomly allocated to the various treatment groups as described in chapter 2 section 2.1. On Day 5 on *in vitro* culture, blastocysts hatching away from the ICM underwent trophectoderm biopsy (as detailed in chapter 2 section 2.4.2). Hatched blastocysts were not biopsied due to difficulty holding them stationary without the zona pellucida. Using Garner and Schoolcraft's blastocyst grading system (section 2.3.2.2) these are all blastocysts graded between 5AA and 5CC. Un-stimulated (n=12), PMSG (n=9), 0.5IU hMG (n=11), 5IU hMG (n=11), 0.5IU Pergoveris (n=10) and 5IU Pergoveris (n=12) biopsied samples were chosen at random for imprinted gene analysis, so as not to introduce bias. Randomly chosen trophectoderm biopsies were also selected from the un-stimulated, PMSG, 0.5IU hMG and 5IU Pergoveris treatment groups to assess RAS gene expression (n=12 samples per group). They were designated for polymerase chain reaction (PCR) for gene expression analysis (Section 2.4.3).

5.2.1 Statistical analyses

Data was tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests using SPSS version 16 (IBM Software Services, Hampshire, UK). Statistical relevance was set at $P<0.05$ for all analyses. Gene expression was not normally distributed and could not be successfully transformed so was graphically represented by a box plot and analysed using the non-parametric Kruskal-Wallis test on SPSS. Any

sample with an 18s Ct value above 26 was omitted due to lack of biological significance.

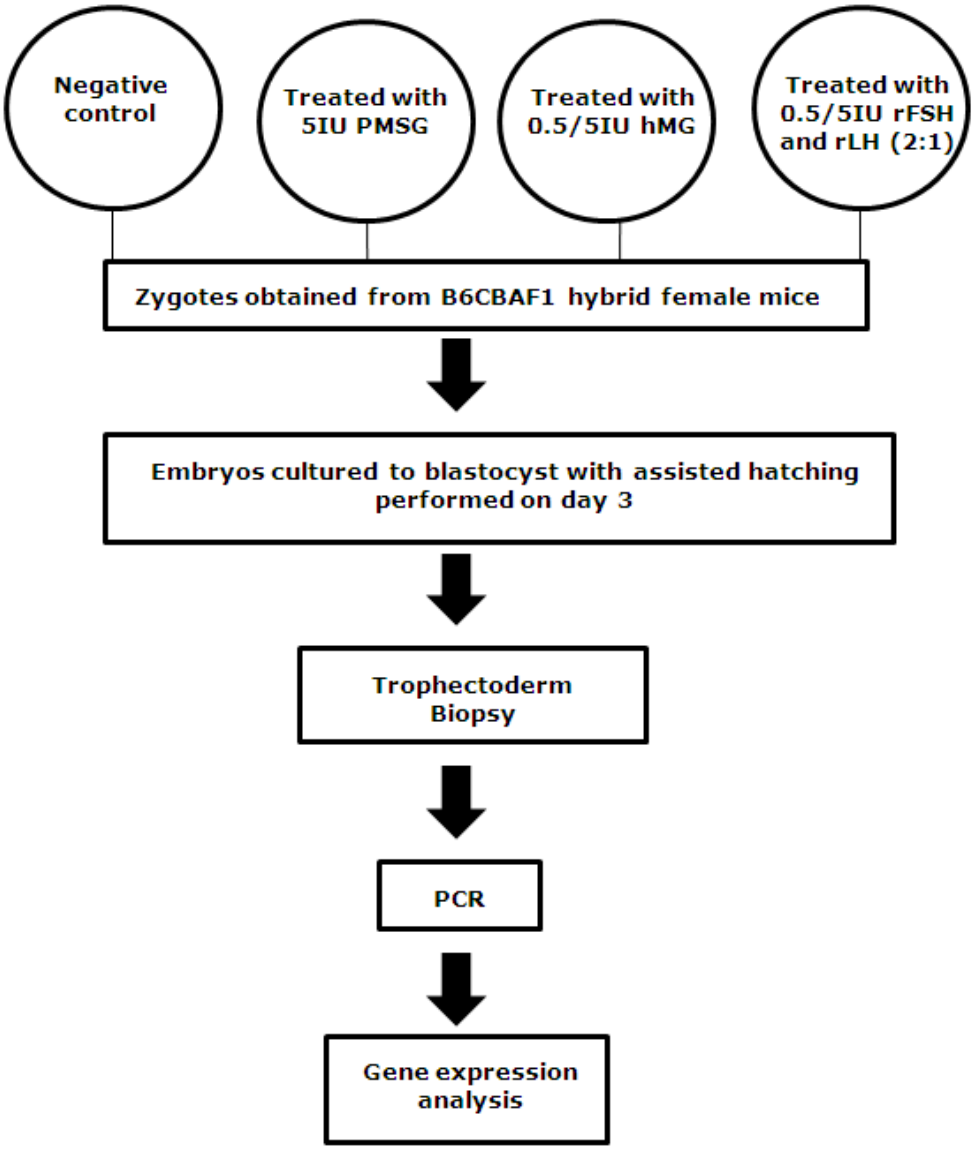

Figure 5.1: Flow chart summarising the experimental design used in this chapter.

5.3 RESULTS

5.3.1 Imprinted gene expression

5.3.1.1 H19 expression

H19 gene expression fold differences were detectable in low levels in the treatment groups. The median fold difference for all the treatment groups was 0. Expression was not evident in blastocysts following treatment with 5IU hMG and 0.5IU Pergoveris. However, these trends were not significant (P>0.05, Figure 5.2).

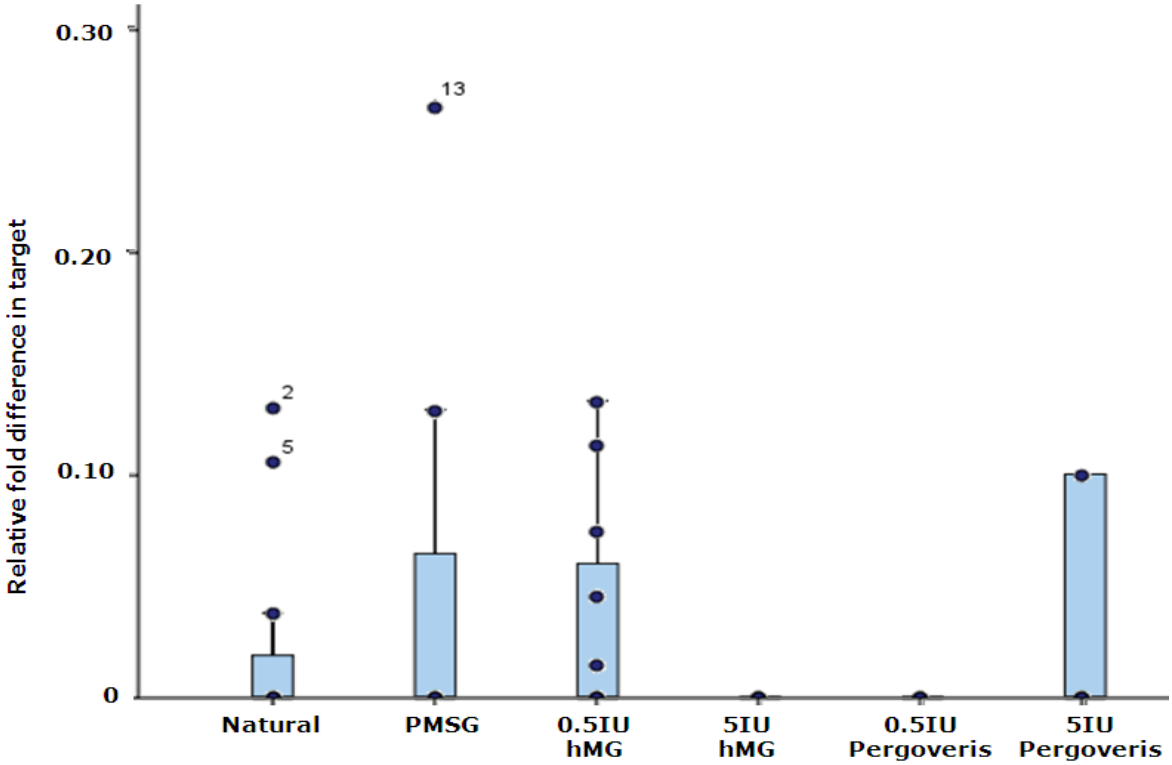

Figure 5.2: Median relative fold difference of *H19* transcript levels in the treatment groups. Error bars depict the inter-quartile range.

5.3.1.2 IGF2 expression

The relative median fold difference of *IGF2* gene expression in the un-stimulated group was 0.56 with an inter-quartile range of 0.7. In contrast, treatment with PMSG and 0.5IU hMG resulted in a 2-3 times decrease in *IGF2* expression which was statistically significant (P<0.05, Figure 5.3). The relative fold difference of 5IU hMG, 0.5IU Pergoveris and 5IU Pergoveris treatment groups was comparable to control (P>0.05).

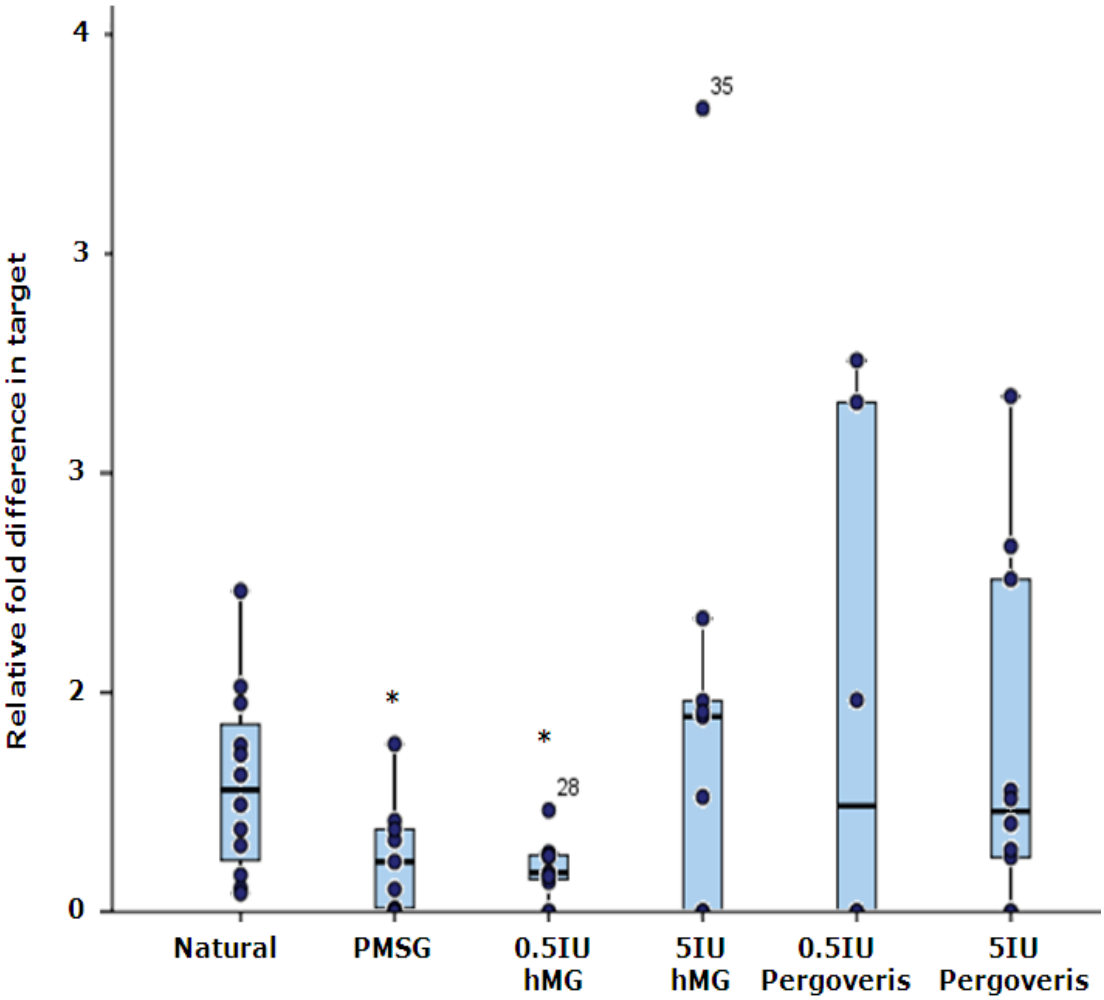

Figure 5.3: Median relative fold difference of *IGF2* transcript levels in the treatment groups. (* P<0.05 compared to natural as assessed by Mann-Whitney U test). Error bars depict the inter-quartile range.

5.3.1.3 IGF2r expression

The relative fold differences of the *IGF2r* mRNA transcripts were equivalent between the ovarian stimulation groups when compared to the natural control (P>0.05). As graphically represented in Figure 5.4, PMSG had the highest inter-quartile range.

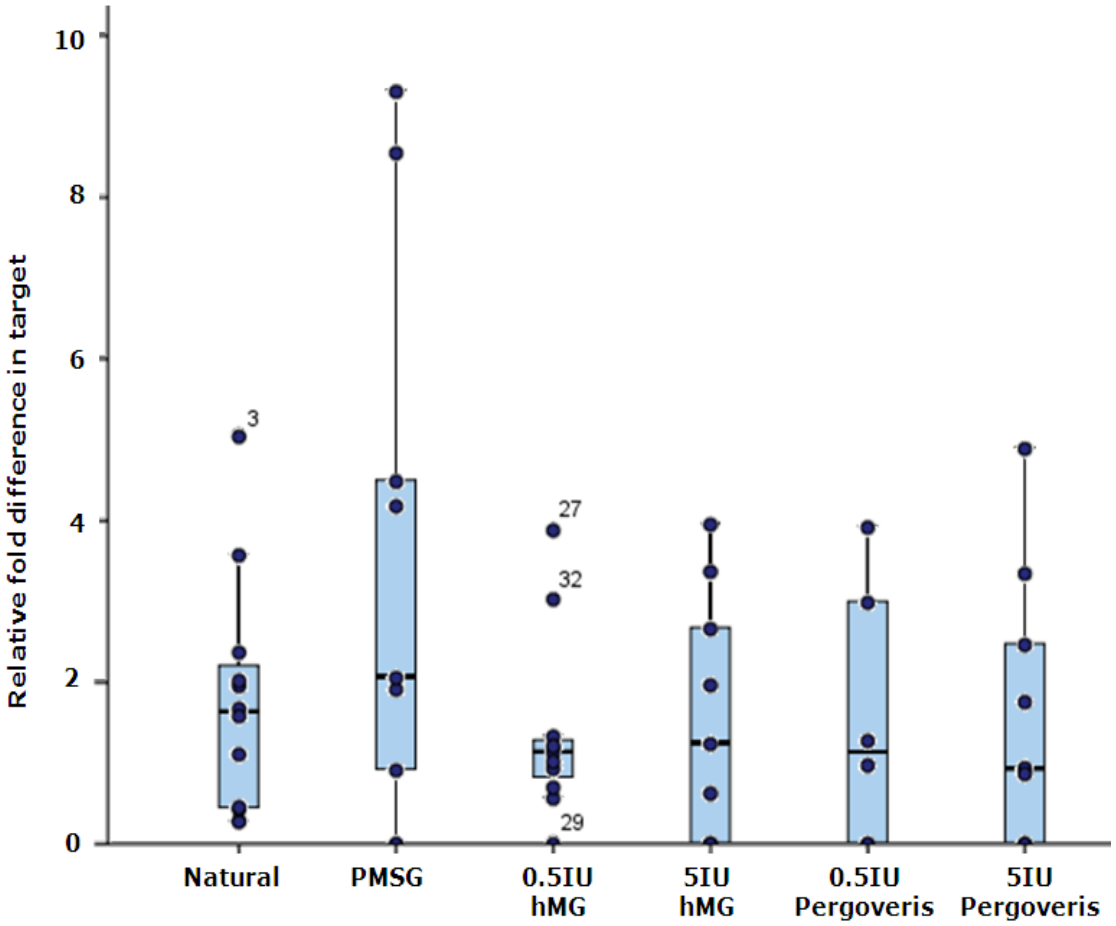

Figure 5.4: Median relative fold difference of *IGF2r* transcript levels in the treatment groups. Error bars depict the inter-quartile range.

5.3.2 Examination of the RAS system

5.3.2.1 ATR1 expression

The relative median fold difference of *ATR1* gene expression in the un-stimulated control group was 1.34 with an inter-quartile range of 5.71 (Figure 5.5). Blastocysts following treatment with PMSG had comparable expression to natural controls (P>0.05) whereas expression in the 0.5IU hMG and 5IU Pergoveris groups was 2.5-3 fold greater than the naturally ovulated group, however these trends were not statistically significant (P>0.05).

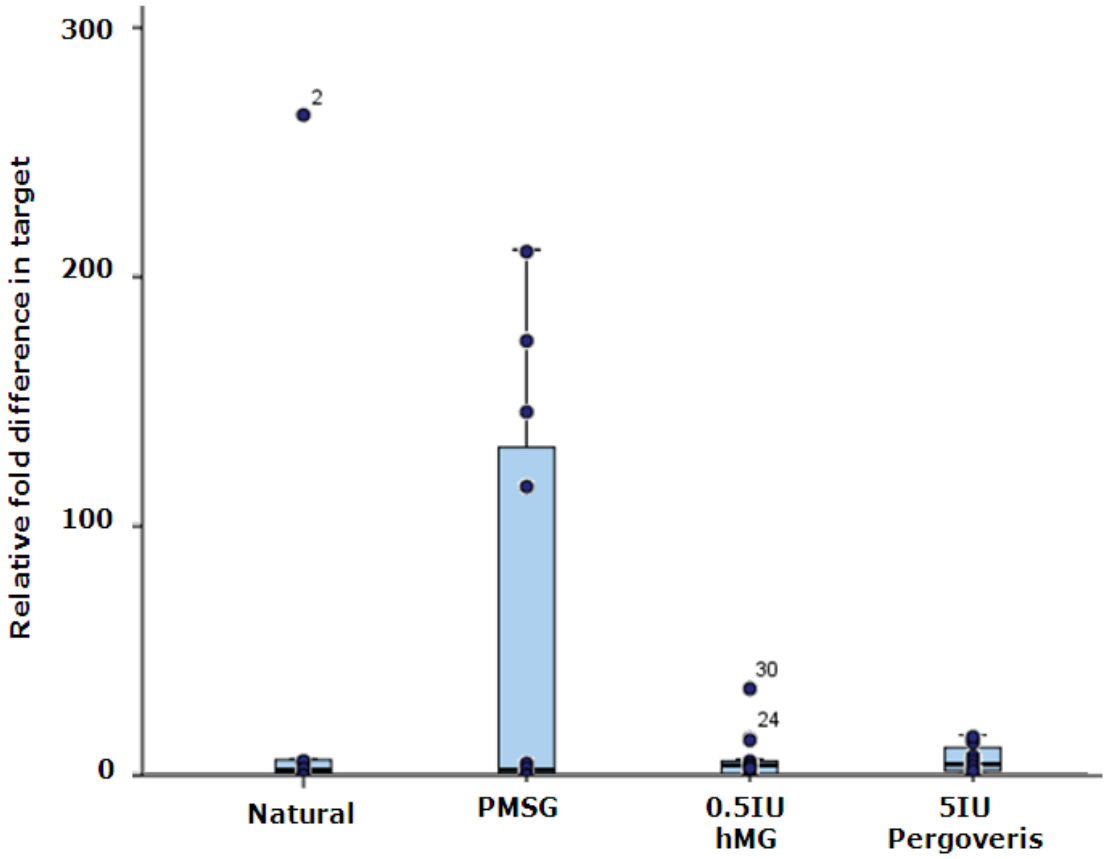

Figure 5.5: Median relative fold difference of *ATR1* transcript levels in the treatment groups (n=12 for all). Bars show the inter-quartile range.

5.3.2.2 ATR2 expression

ATR2 was not detected in any of the biopsied samples selected for analysis. Furthermore, with reducing concentration on the blastocyst pool standards, *ATR2* became undetectable.

5.3.2.3 VEGFA expression

The relative median fold difference of *VEGFA* gene expression in the naturally cycling control blastocysts was 4.3±4.13. The number of *VEGFA* mRNA transcripts was diminished 6.5 fold in the following treatment with PMSG ($P<0.01$, Figure 5.6). The relative fold difference of 0.5IU hMG treated blastocysts was also reduced 5 fold although this trend was not statistically significant ($P>0.05$). The 3.5 fold decline of mRNA transcript levels in the 5IU Pergoveris group was significantly different compared to the natural group ($P<0.05$).

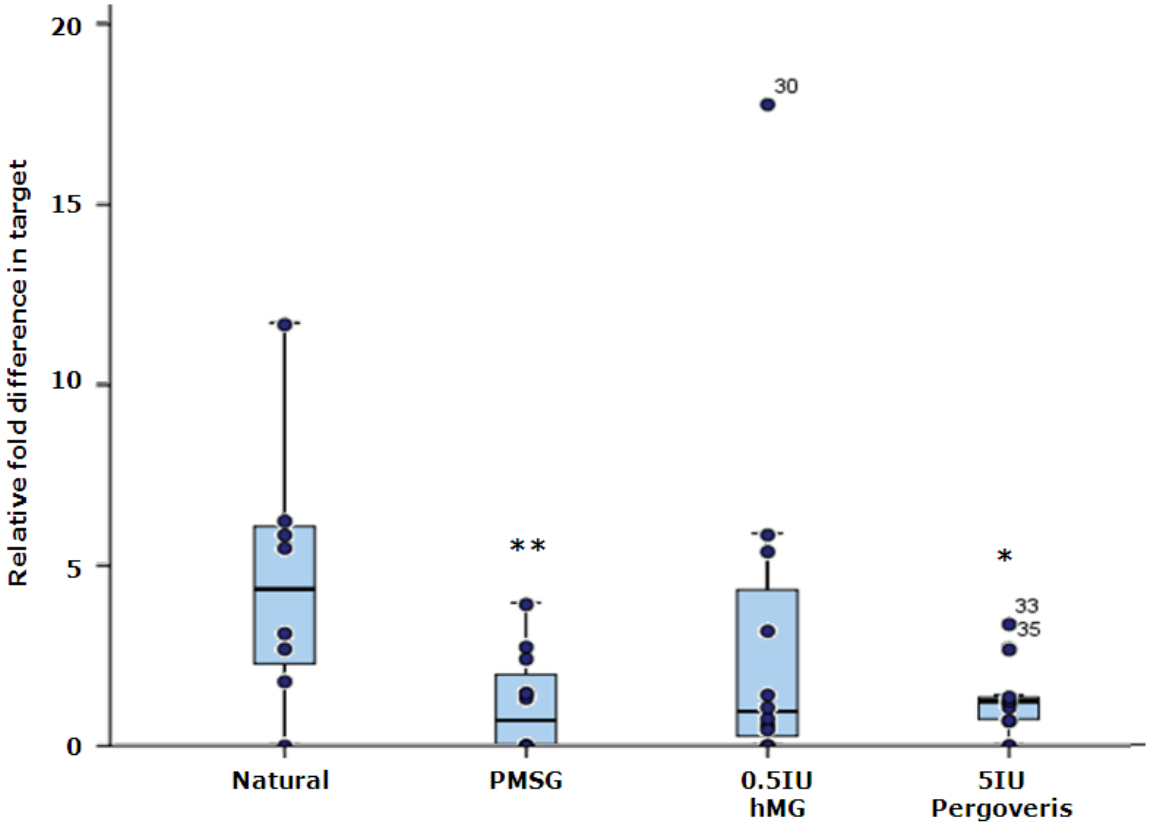

Figure 5.6: Median relative fold difference of *VEGFA* transcript levels in the treatment groups (n=12 for all). (* $P<0.05$ and ** $P<0.01$ compared to control as assessed by Mann-Whitney U test).

5.4 DISCUSSION

In this study it was hypothesised that the use of human gonadotrophin preparations for ovarian stimulation in mice would not be as disruptive to imprinted gene expression as preparations containing long acting LH (PMSG/hMG), based on previous oocyte and embryo development analyses (Chapter 3 and 4). The results obtained suggest that different stimulation regimes result in differences in the pattern of expression of some imprinted and angiogenic genes, which may be associated with LH bioactivity and stimulation dose.

5.4.1 Ovarian stimulation alters *IGF2* gene expression

In this study, ovarian stimulation with PMSG and low doses of hMG resulted in a decrease in *IGF2* mRNA levels. Previous reports suggested *IGF2* expression in the mouse may switch at the blastocyst stage from being only paternally expressed to being bi-allelically expressed (Szabó and Mann, 1995; Li *et al.*, 2005; Ohlsson *et al.*, 1993). Therefore it may be that blastocysts from the PMSG and low dose hMG groups may have their expression reduced relative to this. This may be due to incorrect imprint establishment as a consequence of the superovulation protocols. In conflict with other methylated imprinted genes, the hyper-methylated regions on the paternal *IGF2* allele are expressed and the maternal hypo-methylated DMR repressed (Sasaki *et al.*, 1992). Reduced expression of *IGF2* may be caused by a loss of methylation at the paternal *H19/IGF2* DMR (Thompson *et al.*, 2001). Treatment with 5IU hMG resulted in increased *IGF2* expression, however this was not significant. This could be due to the low numbers analysed.

The *IGF2-H19* gene complex plays a major role in the nutrient transfer capacity of the placenta (Tycko and Morison, 2002). *IGF2* modulates the expression and activity of nutrient transporters, therefore its over- or under-expression may have consequences for foetal growth (Constância *et al.*, 2002). The association of reduced *IGF2* gene expression with intra-uterine growth retardation (IUGR) (Grandjean *et al.*, 2000) and Wilms

tumour, a childhood cancer of the kidney, in addition to other forms of benign and malignant cancers (Ravenel *et al.*, 2001; McCann *et al.*, 1996; McMinn *et al.*, 2006), highlights the importance of further research to explore the biological consequences of reduced *IGF2* expression during pregnancy and resulting offspring observed following stimulation with PMSG and 0.5IU hMG.

Two cell mouse embryos obtained after superovulation with PMSG have been previously shown to display twice the frequency of methylation imprint perturbations compared with those naturally ovulated which in addition showed impeded embryo development and embryo loss during implantation (Shi and Haaf, 2002). Embryo development was also adversely affected by superovulation in our studies (Chapter 3). However, the observation that not all superovulated blastocysts had aberrant *IGF2* gene expression in our study may be due to the later stage of analysis, at the blastocyst stage. As embryos capable of developing to the blastocyst stage are considered to have better developmental potential and therefore only the better quality embryos out of each treatment group was analysed. A previous study reported methylation perturbations in arrested embryos of patients following IVF , however cryopreserved blastocysts donated for research several years later from the same patients were foud to be normal (Ibala-Romdhane *et al.*, 2011). Perturbations in gene expression have also been correlated with blastocyst morphology (Fauque *et al.*, 2007); therefore selectively only assessing hatching blastocysts for trophectoderm biopsy in our study, which Fauque *et al* suggested had the highest proportion of normally methylated embryos, may also have led to similar gene expression between the treatment groups. The observations suggest that embryos after ART which undergo developmental arrest during culture may do so in part due to imprinting defects (van Montfoort *et al.*, 2012). Further research will need to be undertaken to confirm this.

Disruption of *H19* and *IGF2* expression was previously observed in individual blastocysts and in later gestation of 9.5 day post coitum (dpc) placentas in mice conceived after superovulation with PMSG (Fauque *et*

al., 2007). These findings suggest that methylation perturbations can be propagated through the cell lines and indicate that the trophectoderm cell lineage may be less robust at imprint maintenance (Fortier *et al.*, 2008). Reduced expression of *H19* and *IGF2* has also been found to be reduced in human placental tissue following ART (Turan *et al.*, 2010). This may possibly be due to the exposure of these peripheral cells to the external environment of either the uterine milieu or embryo culture system (Fortier *et al.*, 2008; Mann *et al.*, 2004). Many embryos after superovulation have now been demonstrated to harbour aberrant methylation for two or more genes however suboptimal *in vitro* culture conditions and the invasive nature of oocyte collection and embryo transfer could also contribute to methylation defects (Fauque *et al.*, 2007).

In our study we only observed differences in *IGF2* expression between hMG and PMSG compared to the unstimulated control. This could be attributed to the low numbers analysed as a result of cost constraints or due to the analysis of embryonic gene expression in our study being performed on trophectoderm biopsy samples. This may have potentially dampened the negative consequences of ovarian stimulation on gene expression as embryos capable of developing to the blastocyst stage especially hatching are considered to have better developmental potential. Perturbations in gene expression have also been correlated with blastocyst morphology with hatching blastocysts shown to have the highest of proportion of normally methylated embryos (Fauque *et al.*, 2007). However these would be the embryos of choice for transfer by the clinical embryologist if the patient had them in their developing cohort. In our study only the mural trophectoderm cells, i.e. those furthest away from the ICM were analyzed. It is these cells which differentiate into primary trophoblast giant cells during implantation (Negrón-Pérez *et al.*, 2013), therefore we cannot definitively conclude that the gene expression in this region is comparable with the rest of the blastocyst, which is what has been assessed in previous studies.

5.4.2 Ovarian stimulation alters *H19* gene expression

H19 gene expression in the present study was detected at low levels in all the treatments groups, including un-stimulated controls. This maternally expressed gene has four DNase-1 hypersensitive sites which are coated by nuclear proteins to protect the DMR from becoming re-methylated (Feil and Khosla, 1999). Methylation of this region results in complete absence of *H19* expression (Thompson *et al.*, 2002). Abnormal methylation of the *H19* DMR has previously reported following ovarian stimulation with PMSG in a dose dependant manner (Market-Velker *et al.*, 2010). Blastocysts following treatment with 5IU hMG had a complete absence of *H19* expression, whereas the lower dose had comparable expression to un-stimulated controls ($P>0.05$). Absence of expression was also evident in the 0.5IU Pergoveris treatment group. Both these groups had poor embryo development and blastocyst quality (Chapter 4), which may provide some support for the theory, that perturbed development may be due to imprinted defects (van Montfoort *et al.*, 2012).

New evidence suggests it is not the initial imprints which are affected by ovarian stimulation, but insufficient maternal mRNA stores through incomplete oocyte maturation (Li *et al.*, 2010). The hypothesis that ovarian stimulation results in disruption of maternal effect gene products which are required to maintain correct imprinting throughout embryo development is based on the observation that MII mouse oocytes collected after ovarian stimulation had comparable methylated *H19* gene expression compared to un-stimulated controls (Denomme *et al.*, 2011), however the same research group had previously shown that *in vivo* collected blastocysts after the same regime showed imprint perturbations (Market-Velker *et al.*, 2010). It may be that *H19* gene expression was not significantly different in our study due to all treatment groups only being analysed at the hatching blastocyst stage. To remove this potential bias it may have been more appropriate to assess gene expression of whole blastocysts, either individually or pooled at different classification stages for example expanded, hatching and hatched blastocysts or even earlier.

The analysis of whole blastocysts would also prevent genetic perturbations remaining unidentified as a consequence of differential gene expression between the ICM and TE (Ozawa *et al.*, 2012).

5.4.3 Ovarian stimulation alters RAS gene expression

ATR2 expression was absent in all trophectoderm biopsies as expected due to this receptors mediated effects of cell proliferation and vasodilatation inhibition (Nielsen *et al.*, 2000). Angiotensin receptor 1 (AT_1) on the other hand causes vasoconstriction and promotes growth processes required throughout pregnancy and is the predominate angiotensin receptor localised in the extra villous trophoblast (EVT) (Kalenga *et al.*, 1996; Li *et al.*, 1998). AT_1 expression was comparable for all the ovarian stimulation regimes and control; however blastocysts following treatment with PMSG did show the greatest variation in expression levels. In the rat, the RAS system is suggested to be involved in differentiation of stromal cells into decidual cells during decidualisation which is a pre-requisite for implantation of the blastocyst (Squires and Kennedy, 1992), most likely through the actions of the AT_1 receptor (Tower *et al.*, 2010). Disruptions in the RAS has been implicated in cases of pre-eclampsia and IUGR, specifically down regulation of AT_1 receptors in the placenta (Li *et al.*, 1998). Over-expression of AT_1 may in theory result in the opposite effect, causing overgrowth of the foetus.

The 5IU PMSG and 5IU Pergoveris groups had lower expression levels of *VEGFA*, compared to control. Blastocysts following treatment with 0.5IU hMG had comparable expression. Decidua formation during implantation has been associated with an increase in maternal angiogenesis regulated by *VEGFA* (Douglas *et al.*, 2009) and hypothesised to originate from the EVT of the embryo (Plaisier *et al.*, 2007). The lower expression levels observed in the ovarian stimulation treatment groups may explain why less of the transferred blastocysts implanted, resulting in a live birth. Lower implantation rates following ovarian stimulation have consistently been observed in mice (Van der Auwera and D'Hooghe, 2001; Ghaemi *et*

al., 2008; Fossum *et al.*, 1989; Ertzeid and Storeng, 1992; Ertzeid and Storeng, 2001; Ertzeid *et al.*, 1993).

5.4.4 Conclusion

The results of this study show some perturbation in gene expression which does support the initial hypothesis that ovarian stimulation does disrupt expression of imprinted and angiogenic genes. However, more work is needed with a greater number of repeats, different stages of analysis throughout embryonic development and a wider range of genes assessed (gene array).

CHAPTER 6: THE EFFECT OF OVARIAN STIMULATION ON PREGNANCY AND DEVELOPMENT OF LIVE YOUNG

6.1 INTRODUCTION

Adverse effects of ovarian stimulation using urinary and recombinant preparations in humans have been reported. Singleton conceptions generated through IVF have been associated with more peri-natal complications than naturally conceived counterparts, such as pre-term birth, low birth weight, intra-uterine growth restriction (IUGR) and postnatal health issues requiring hospital admission (Jackson *et al.*, 2004; Helmerhorst *et al.*, 2004; Basatemur and Sutcliffe, 2008). However these studies analysed the effects of superovulation as a whole and did not distinguish between the uses of different gonadotrophins.

It is evident from the HFEA implantation data that approximately 80% of embryos transferred fail to implant in infertile women undergoing IVF, potentially due to the use of ovarian stimulation (Martínez-Conejero *et al.*, 2007). Implantation failure can be a consequence of poor endometrial receptivity or to inherent problems with the embryo (Ertzeid and Storeng, 2001). Studies distinguishing between these two causes are scarce and require further clarification. No previous work has focused on how ovarian stimulation with human gonadotrophins with different FSH and LH half lives has on implantation and subsequent progeny.

This objective of this experiment was to assess the effect of embryo transfer and ovarian stimulation with different gonadotrophins compared with unstimulated cycles, on pregnancy and development of live young. The end points measured were live birth rate and offspring development.

6.2 METHODS OVERVIEW

Mice were randomly allocated to the various treatment groups as described in chapter 2 section 2.1 and following *in vitro* culture, blastocysts were transferred to pseudo-pregnant recipients:

Table 6.1: Number of mice in each treatment group from which zygotes were collected from and blastocysts transferred to. (The technical number of repeats for each group was n=2).

Treatment	Collection numbers (n)	Transfer numbers (n)
Un-stimulated	20	5
5IU PMSG	12	8
0.5IU hMG	12	8
5IU Pergoveris	12	5

The experimental pathways in this chapter are schematically represented in Figure 6.1.

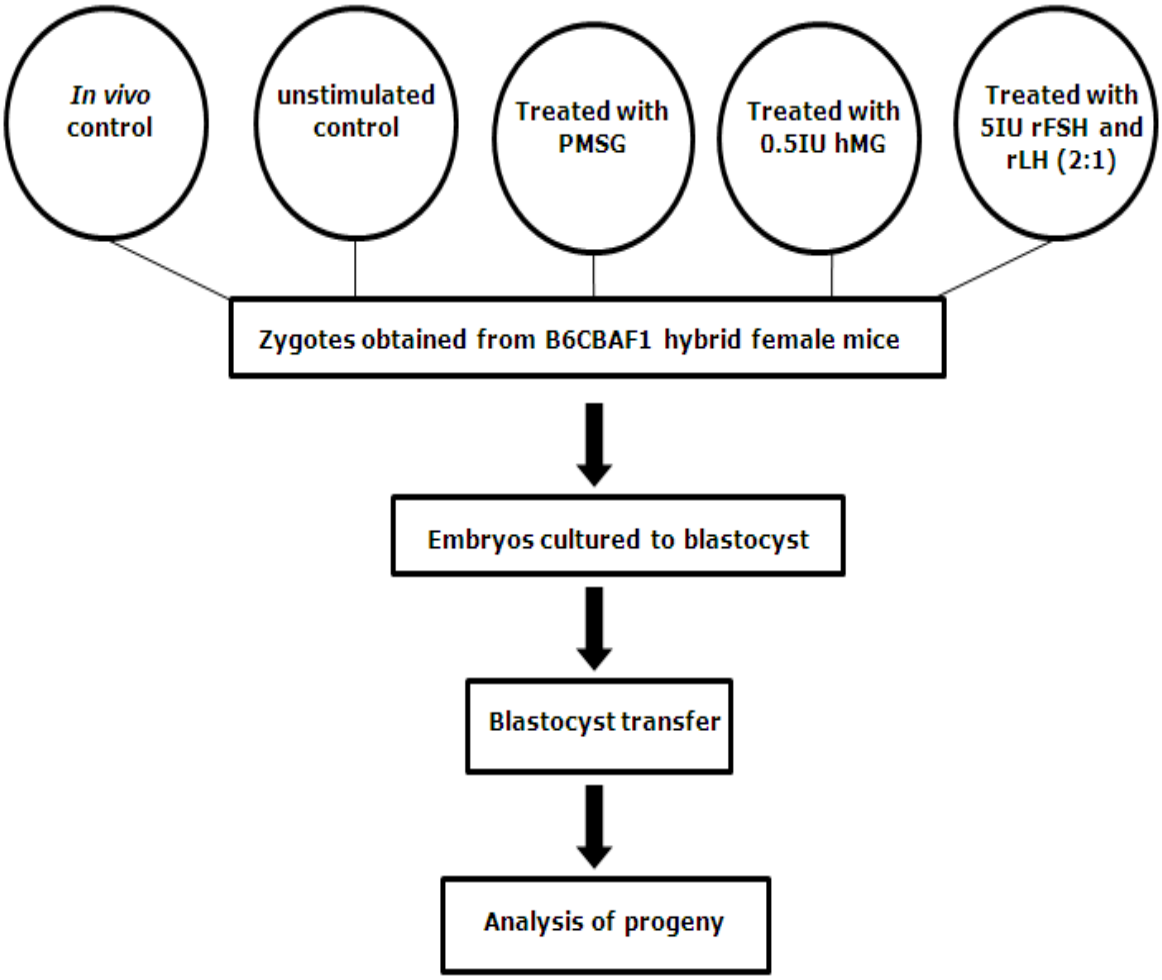

Figure 6.1: Flow chart summarising the experimental design used in this chapter, giving details of the different pathways.

6.2.1 Embryo transfer

Twenty blastocysts per surrogate were transferred between 2-4pm on the 5th day of *in vitro* embryo culture. All blastocysts were randomly transferred to the CD1 embryo recipients to avoid selection bias. A nonsurgical embryo transfer (NSET) device was used. Blastocysts were transferred into 2.5dpc nine week old CD1 pseudo pregnant mice as it has been shown to be an effective method in generating live pups (Green *et al.*, 2009).

6.2.2 Preparation of pseudo-pregnant surrogates

Eight week old CD1 female mice were group housed for one week to synchronise oestrus cycles and then exposed to soiled male bedding to induce ovulation (whittened) for two nights prior to monogamously caging with nine week old vasectomised CD1 males on the third night. CD1 males had been vasectomised at 6 weeks post birth and allowed to recover for 3 weeks to ensure no residual sperm remained. Vaginal plug detection was classed as 0.5dpc of pseudo pregnancy and female mice were housed for two more nights prior to embryo transfer (ET) to reach 2.5dpc.

6.2.3 Non-surgical blastocyst transfer

Blastocysts were transferred to a 15µl drop of pre-equilibrated G-2 media. The number of blastocysts transferred was kept constant at 20 per surrogate. The NSET device was placed on a P2 Gilson pipette and set to 1.8µl. After blastocysts were loaded into the device the volume was adjusted to 2µl to create a small air bubble at the tip to prevent the embryos wicking on insertion. The female was permitted to grasp the bars of a new cage and angled in a vertical position by grasping the base of the tail. The small speculum was first inserted into the vagina to stretch it before inserting the larger speculum. After visualisation of the cervix, the NSET device was inserted all the way into the cervix, parallel to the spine, until the NSET hub made contact with the speculum. The blastocysts were expelled and the NSET device removed from the animal without releasing the plunger. The device was subsequently checked under a microscope to ensure expulsion of all the blastocysts. If blastocysts remained in the device, these were discarded and the actual number of blastocysts transferred documented. The NSET device directly deposits the blastocysts into the uterine horn, near the oviduct as demonstrated by the injection of Coomassie blue during training (Figure 6.2).

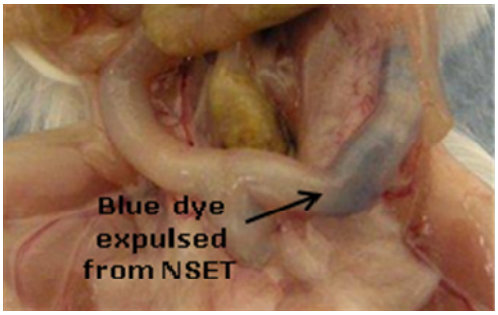

Figure 6.2: Location of blue dye in a single uterine horn following use of the NSET device.

6.2.4 Assessment of live offspring

Females were housed in pairs and after 15 days post transfer, females which showed outwards signs of pregnancy were separated. The pregnancy rate per treatment was calculated. A selection of plugged B6CBAF1 females were allowed to deliver young for use as *in vivo* controls for the offspring analyses.

6.2.4.1 Number of days gestation

Mice were checked morning and evening for litters and the number of days to parturition was calculated to the nearest 0.5 day as:

Date of birth – Date of transfer (+4 days) = Gestation length

Only 4 days for culture was added as *in vitro* culture is delayed by approximately one day compared to embryos developing *in vivo*.

6.2.4.2 Number of live offspring per treatment

All females were allowed to develop to term to permit assessment of offspring. The number of progeny per surrogate was noted and used to calculate the mean number of offspring obtained per ET and the percentage of offspring per ET, calculated as:

Number of offspring per ET (%) = mean number of offspring obtained per ET / Mean number of embryos transfered

6.2.4.3 Weight trajectory 3 weeks post birth

A selection of male and female pups from each treatment group were randomly selected on Day 10 postpartum, culled by CO_2 inhalation and weighed individually to calculate total body mass. This was repeated at Day 17 and Day 24 to enable a weight trajectory prior to weaning to be generated.

6.2.4.4 Organ morphometry

Euthanized mice were analysed for body composition. Heart, lung, liver, kidney, spleen and pancreas were dissected out of individual mice over a 3 week period. Internal organs were individually placed into pre-weighed sterile 1.5ml centrifuge tubes and kept on ice until weighed using a fine balance scale. Organ weights were made relative to total body mass using the following calculation:

Relative organ weight = Weight of organ / Total body weight

6.2.5 Statistical analyses

Data was plotted for histogram and tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests using SPSS version 16 (IBM Software Services, Hampshire, UK). Statistical relevance was set at $P<0.05$ for all analyses. Pseudo-pregnant mice which had an off centre cervix, making embryo transfer difficult were excluded from the analyses, as no pregnancy resulted in these females. Dichotomous data such as pregnancy rates were analysed by chi-squared on Graph Pad prism. Weight trajectory and organ growth analyses of offspring were performed by ANOVA using SPSS.

6.3 RESULTS

6.3.1 Live birth rate and offspring analyses

6.3.1.1 Pregnancy rate

On Day 15 post transfer, 60% of surrogates transferred with natural blastocysts showed outward signs of pregnancy. Surrogates carrying embryos derived from PMSG and 0.5IU hMG treated mice all showed noticeable abdominal swelling (P<0.001). Only half of 5IU Pergoveris surrogates became pregnant. However, there were too few replicates in order to do any statistical analyses for this treatment group.

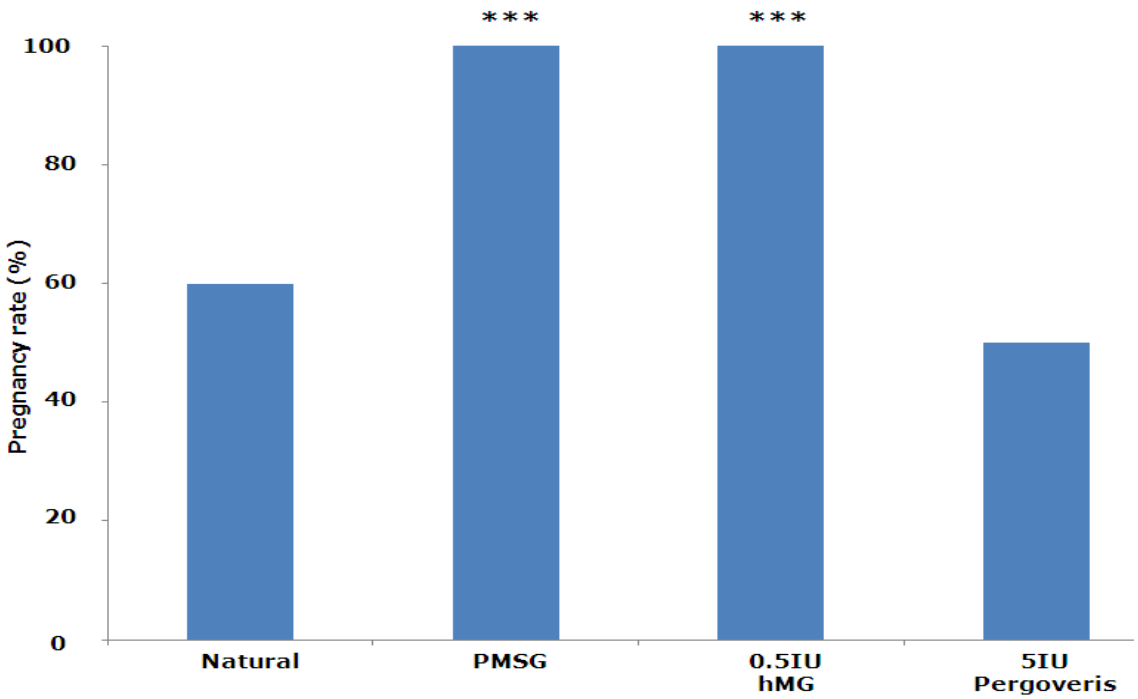

Figure 6.3: Mean pregnancy rates on day 17 gestation for the natural (n=4), PMSG (n=6), 0.5IU hMG (n=4) and 5IU Pergoveris (n=2) treatment surrogates. (*** P<0.001 compared to natural control).

6.3.1.2 Live birth rate

As shown in Table 6.2, the natural cohort had the highest mean live offspring rate with 33%. This was lower following treatment with PMSG, although the trend was not significant (P>0.05). Both the 0.5IU hMG and 5IU Pergoveris ovarian stimulation groups had a lower proportion of the embryos transferred resulting in live offspring (P<0.05). The average litter size was equivalent for all treatment groups (P>0.05).

6.3.1.3 Length of gestation

B6CBAF1 females are reported to take 18.5-21 days to complete gestation. Length of pregnancy for the embryo transfer groups was a day shorter than those naturally pregnant (Table 6.3). When the time of gestation was corrected for the time spent in embryo culture, the total time from fertilisation to delivery of live young was significantly longer for all ET groups in comparison to *in vivo* controls from the day of copulation plug detection (P<0.05).

Table 6.2: Overview of embryo development and pregnancy for the treatment groups. (* P<0.05 and *** P<0.001 compared to natural control). Data is expressed as actual number (% of total).

Treatment	Mean number of zygotes obtained	Mean number of zygotes cleaved (%)	Mean number of blastocysts obtained (%)	Mean number of offspring obtained per ET	Number of offspring per ET (%)
Unstimulated	8	90	85	4	33
PMSG	25	71***	50***	5	24
0.5IU hMG	27	75***	61***	3	18*
5IU Pergoveris	20	53***	50***	3	19*

Table 6.3: Overview of gestation length for the treatment groups.

Treatment	Mean length of pregnancy (Days)	Mean length of gestation including culture (Days)
Natural	17.5±0.5	21.5±0.5
PMSG	17.5±0.5	21.5±0.5
0.5IU hMG	17.5±0.5	21.5±0.5
5IU Pergoveris	17	21
In vivo control	18.5±0.5	18.5±0.5

6.3.1.4 Weekly weight trajectory

There was an expected increase in weight in all treatment groups over the neonatal period of 3 weeks analysed ($P<0.001$, Figure 6.4). Ten day old progeny from the 0.5IU hMG and 5IU Pergoveris treatment groups had a significantly larger total body mass compared to unstimulated ($P<0.001$) and *in vivo* controls ($P<0.001$ and $P<0.01$ respectfully). All embryo transfer group progeny, on day 17, had significantly larger body mass compared to *in vivo* controls, regardless of treatment group ($P<0.001$). When progeny were analysed on day 24, only the 5IU Pergoveris treatment group had significantly larger total body mass compared to unstimulated controls ($P<0.001$).

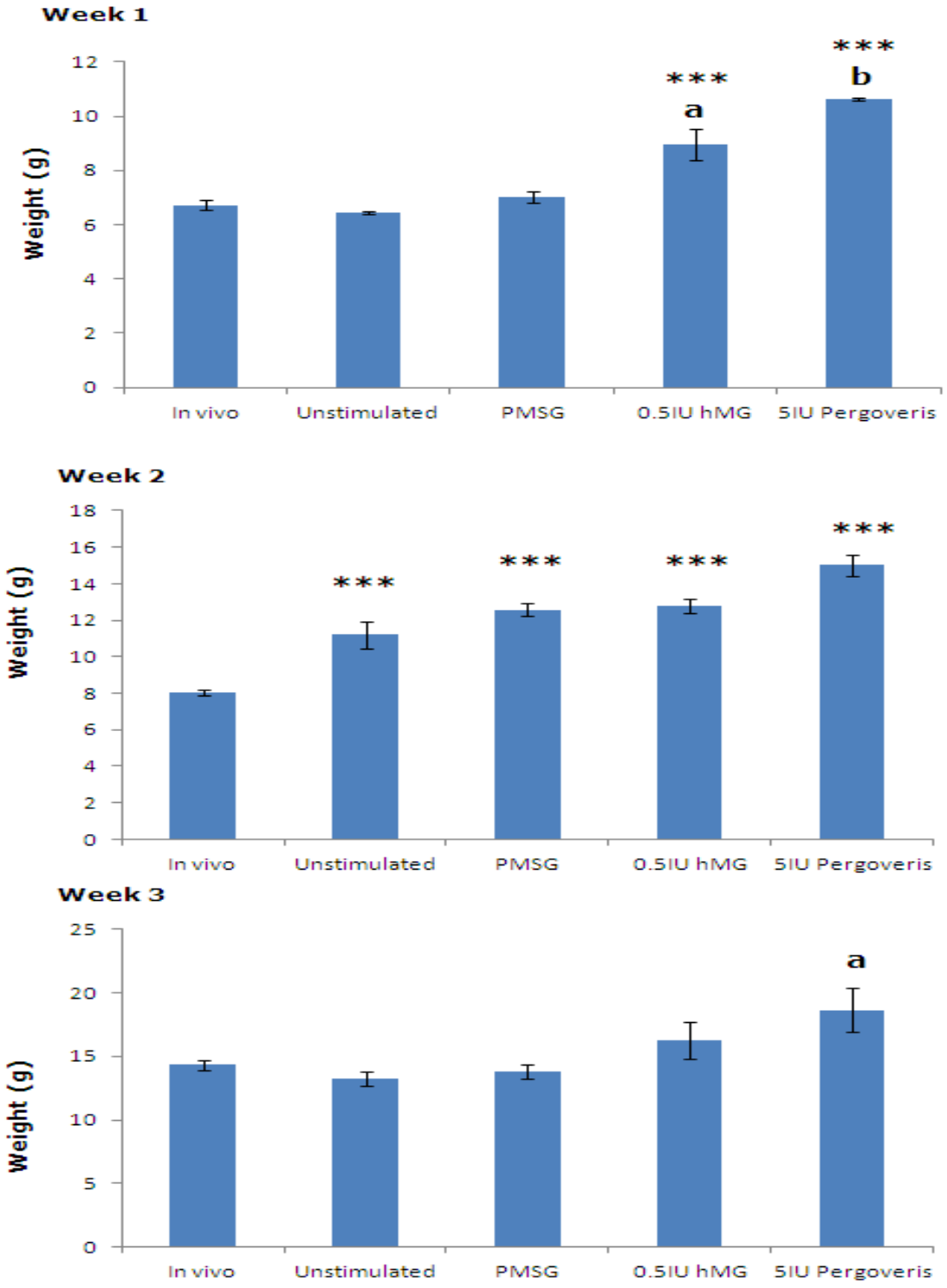

Figure 6.4: Total body mass 3 weeks post birth for *in vivo* (n=31) natural (n=8), PMSG (n=14) 0.5IU hMG litters (n=17) and 5IU Pergoveris progeny (n=6). *** P<0.001 compared to *in vivo* controls. a P<0.001 and b P<0.01 compared to unstimulated controls as assessed by ANOVA.

6.3.1.5 Mean organ weight trajectories

6.3.1.5.1 Heart

Ten day old progeny from the PMSG treatment group had a significantly heavier relative heart mass compared to *in vivo* controls ($P<0.05$, Figure 6.5). On day 17, PMSG progeny had a lighter relative heart mass ($P<0.001$), as did the 0.5IU hMG treatment group, compared to *in vivo* controls. When progeny were analysed on day 24, there was no significant difference in relative heart mass between any of the treatment groups and controls ($P>0.05$). For all weeks analysed there was no significant difference between gonadotrophin treatment groups and unstimulated controls ($P>0.05$).

6.3.1.5.2 Lungs

The first week analysis showed no difference in relative lung mass between any of the treatment groups and controls ($P>0.05$, Figure 6.6). Progeny derived following treatment with PMSG showed a reduced relative lung mass in the second week compared to *in vivo* control progeny ($P<0.001$). In the third week, the relative lung mass was significantly increased for the PMSG and 0.5IU hMG treatment groups ($P<0.01$ for both). For all weeks analysed there was no significant difference between gonadotrophin treatment groups and unstimulated controls ($P>0.05$).

6.3.1.5.3 Liver

There was no significant difference in relative liver weight between any of the treatment groups and controls, for all of the time points analysed ($P>0.05$, Figure 6.7).

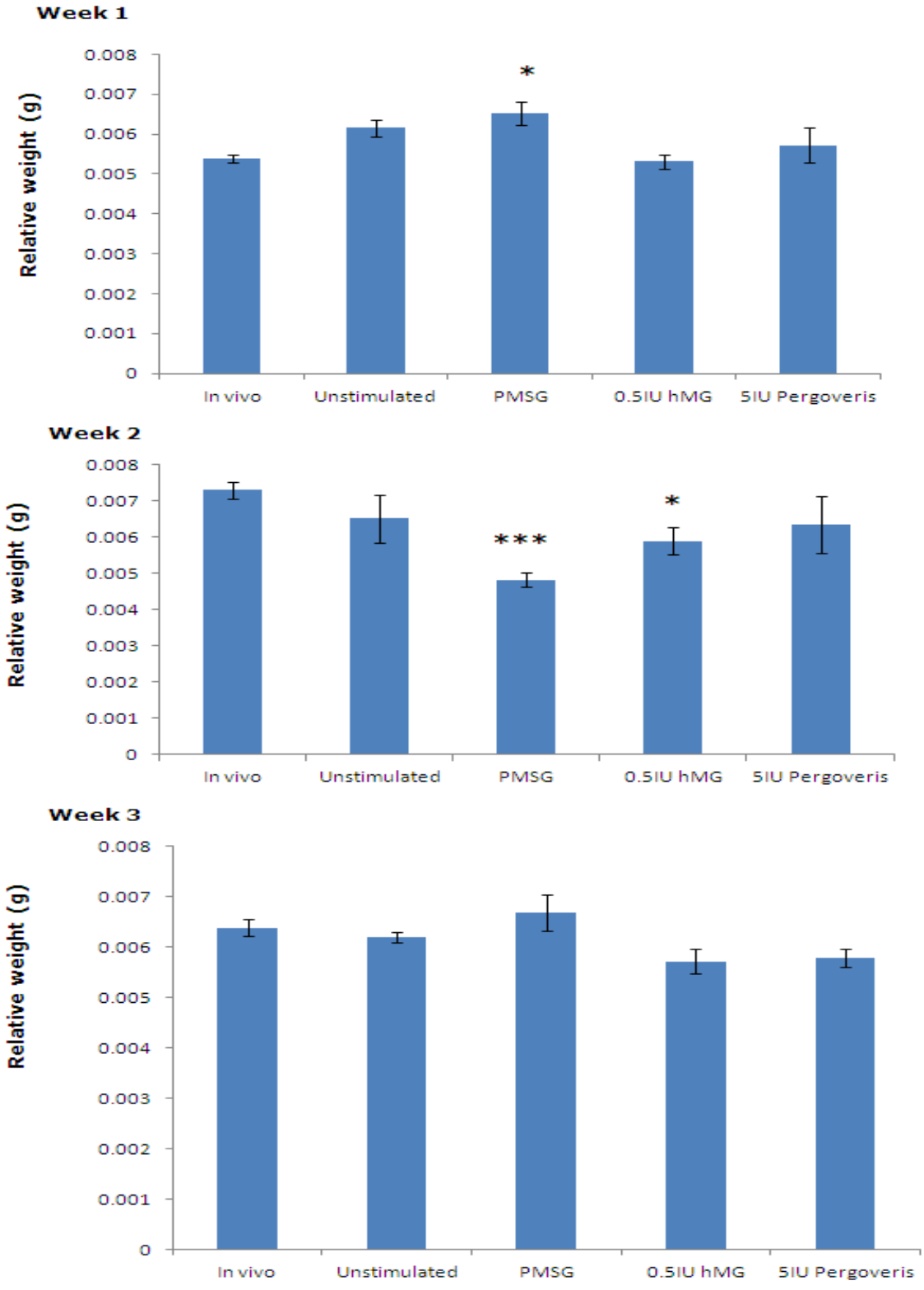

Figure 6.5: Mean relative heart weight 3 weeks post birth for *in vivo* (n=31) natural (n=8), PMSG (n=14) 0.5IU hMG litters (n=17) and 5IU Pergoveris progeny (n=6). *** P<0.001 and * P<0.05 compared to *in vivo* controls as assessed by ANOVA.

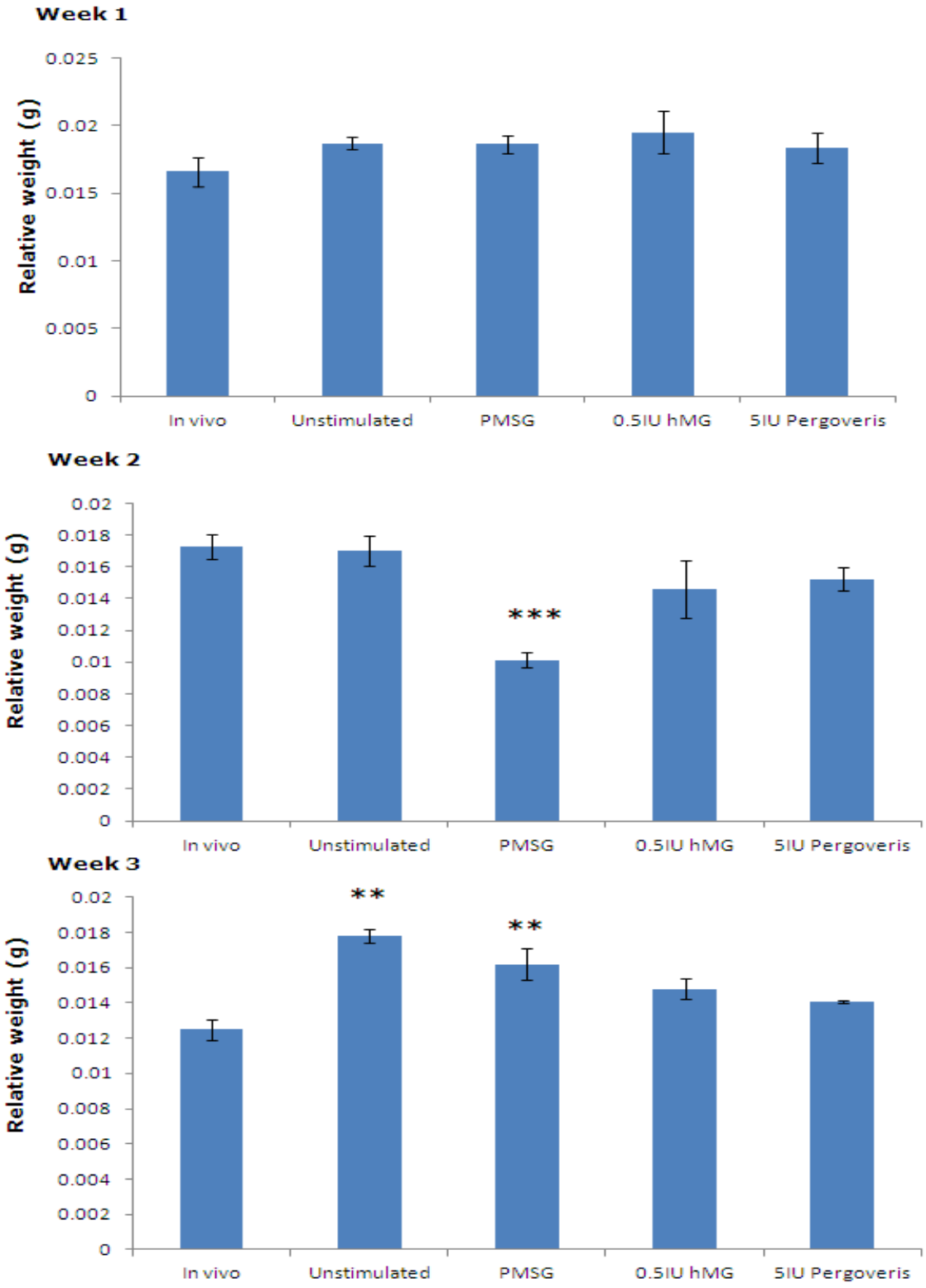

Figure 6.6: Mean relative lung weight 3 weeks post birth for *in vivo* (n=31) natural (n=8), PMSG (n=14) 0.5IU hMG litters (n=17) and 5IU Pergoveris progeny (n=6). *** P<0.001 and ** P<0.01 compared to *in vivo* controls as assessed by ANOVA.

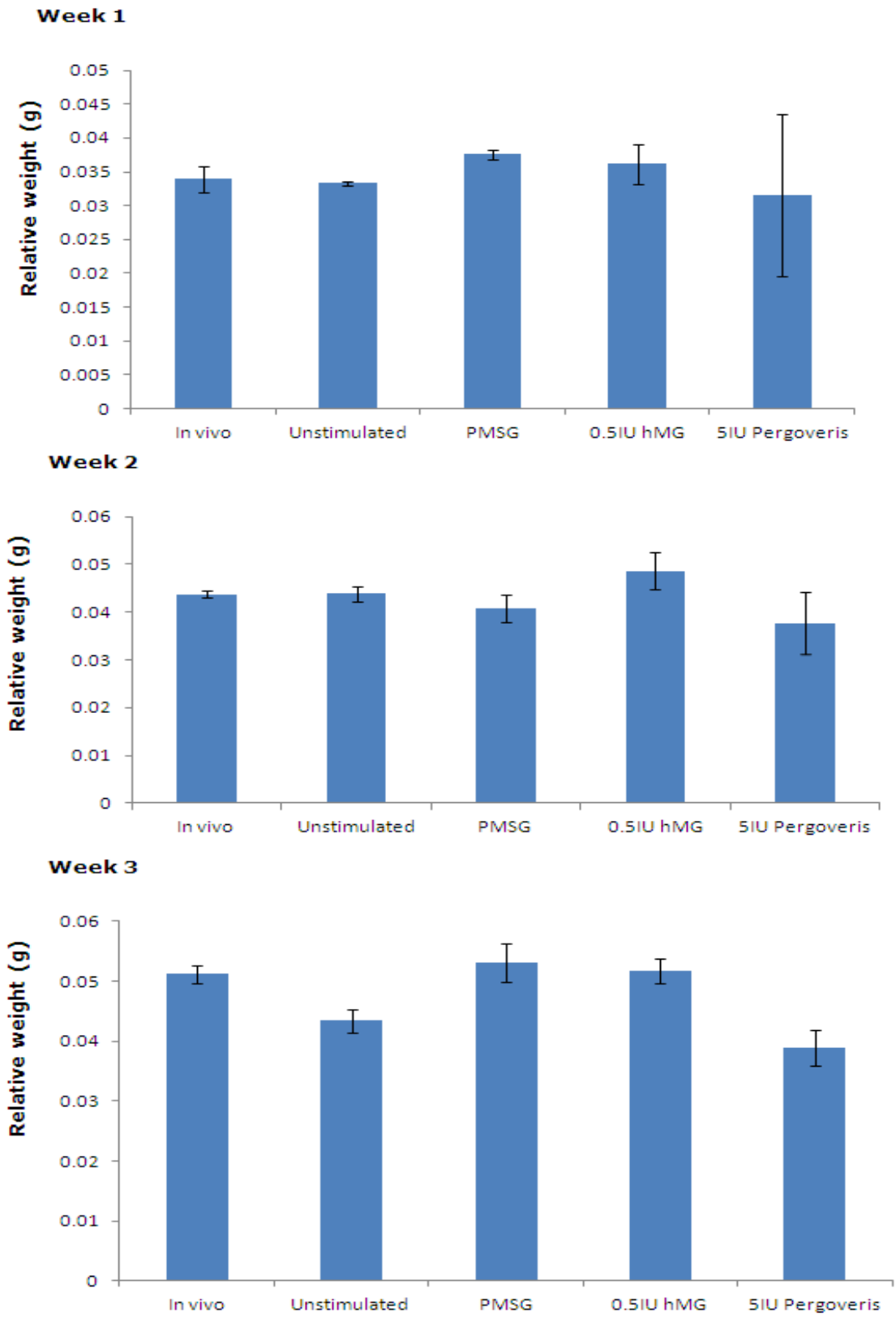

Figure 6.7: Mean relative liver weight 3 weeks post birth for *in vivo* (n=31) natural (n=8), PMSG (n=14) 0.5IU hMG litters (n=17) and 5IU Pergoveris progeny (n=6).

6.3.1.5.4 Kidneys

Ten day old progeny from the PMSG and 0.5IU hMG treatment groups had a significantly heavier relative kidney mass compared to *in vivo* controls ($P<0.05$ and $P<0.01$ respectively, Figure 6.8). On day 17, PMSG progeny had a lighter relative kidney mass ($P<0.05$), compared to unstimulated controls. When progeny were analysed on day 24, there was no significant difference in relative kidney mass between any of the treatment groups and controls ($P>0.05$).

6.3.1.5.5 Spleen

The first week analysis showed no difference in relative spleen mass between any of the treatment groups and controls ($P>0.05$, Figure 6.9). Progeny derived following treatment with 0.5IU hMG and unstimulated controls showed an increased relative spleen mass in the second week compared to *in vivo* control progeny ($P<0.01$ and <0.001 respectively). Offspring from the PMSG treatment group had lighter kidney mass compared to unstimulated controls ($P<0.05$). In the third week, the relative kidney mass was significantly increased for the unstimulated treatment group compared to the *in vivo* control ($P<0.01$). All ovarian stimulation treatment groups had a reduced relative spleen mass compared to the unstimulated control offspring as shown in Figure 6.9.

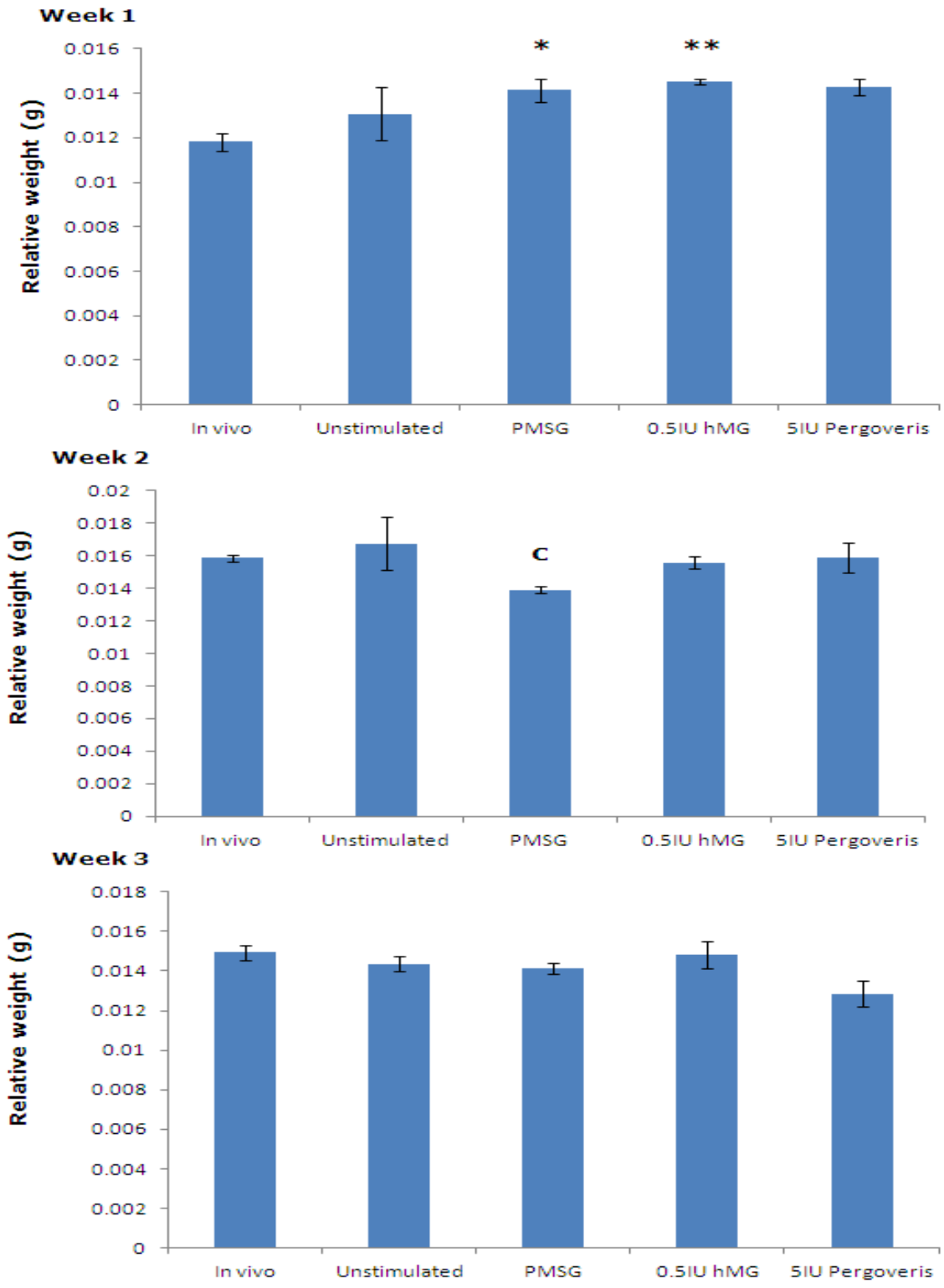

Figure 6.8: Mean relative kidney weight 3 weeks post birth *in vivo* (n=31) natural (n=8), PMSG (n=14) 0.5IU hMG litters (n=17) and 5IU Pergoveris progeny (n=6). ** P<0.01 and * P<0.05 compared to *in vivo* controls and c P<0.05 compared to unstimulated controls as assessed by ANOVA.

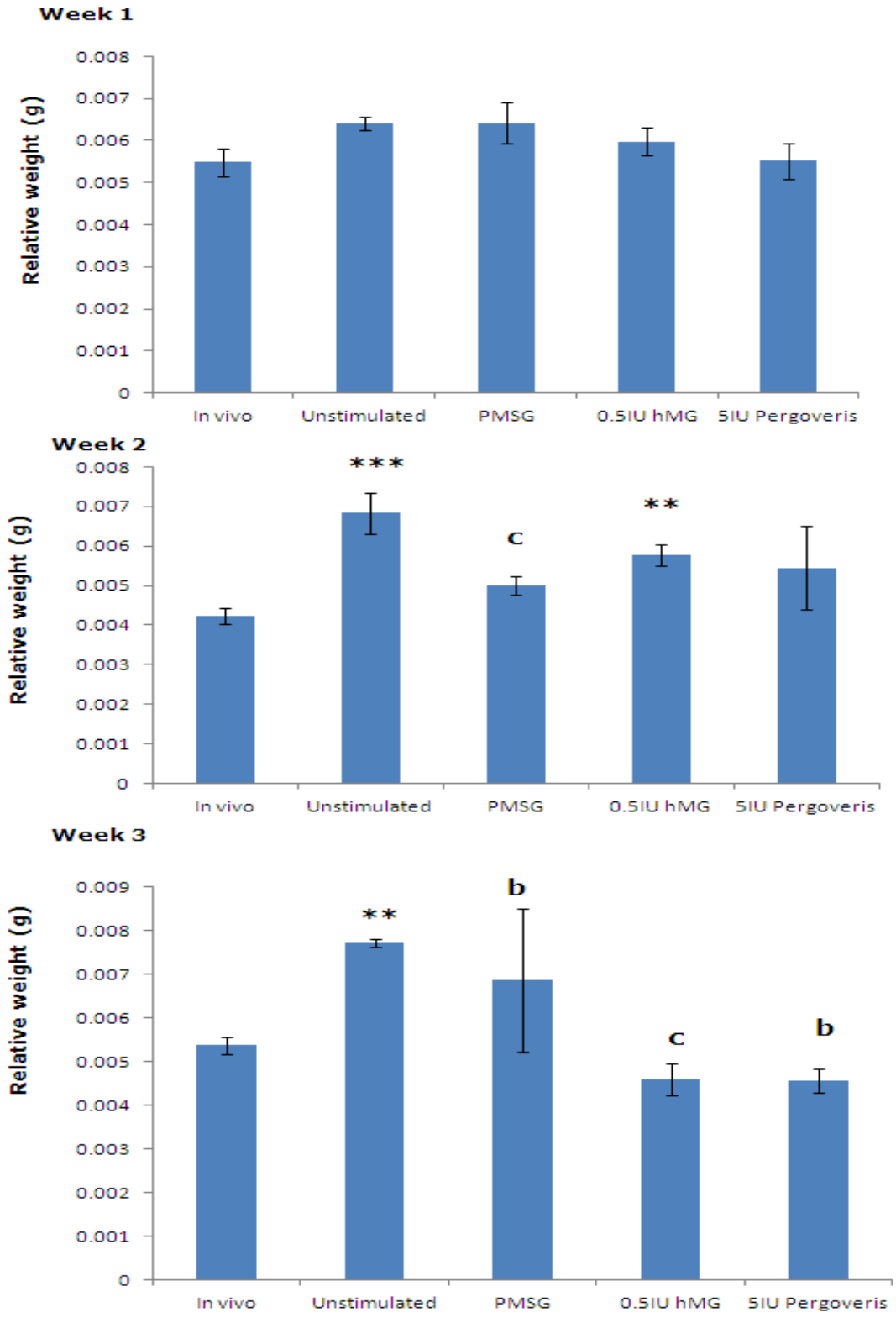

Figure 6.9: Mean relative spleen weight 3 weeks post birth for *in vivo* (n=31) natural (n=8), PMSG (n=14) 0.5IU hMG litters (n=17) and 5IU Pergoveris progeny (n=6). *** P<0.001 and ** P<0.01 compared to *in vivo* controls. b P<0.01 and c P<0.05 compared to unstimulated controls as assessed by ANOVA.

6.4 DISCUSSION

In this study it was hypothesised that the use of human gonadotrophin preparations for ovarian stimulation in mice, based on previously obtained oocyte and embryo development data, would not be as detrimental to pregnancy rates and offspring generated as has been reported for PMSG in the literature. The results obtained suggest that there were more offspring following ET of blastocysts from non-stimulated mice, than for those which were treated with gonadotrophins. In addition, developmental alterations for ovarian stimulated transferred blastocysts were observed, in terms of total body mass and internal organ relative weights. However in some instances the effects observed could have been due to *in vitro* handling. This study provides further information on the effects of ovarian stimulation with different gonadotrophin preparations on disruption of pregnancy and post natal growth.

6.4.1 Superovulation effects pregnancy and live birth rate

The majority of previous studies investigating the effect of ovarian stimulation on subsequent post-implantation development used spontaneously conceiving mouse models. Adverse outcomes for the superovulatory mice may therefore reflect nutritional deficiency as a consequence of overcrowded uteri (Edgar *et al.*, 1987; Evans *et al.*, 1981). This was taken into account in our study by matching the number of embryos transferred in the different treatment cohorts. Reported pregnancy rates post transfer of 80% are comparable to the pregnancy and live birth rates per embryo transfer achieved in our study using the NSET device (Muñoz *et al.*, 1995) and the average litter size reported for spontaneously pregnant PMSG stimulated mice was three (Ertzeid *et al.*, 1993) analogous to our embryo transfer result.

In agreement with previous reports, the live birth rate per embryo transferred of PMSG stimulated embryos was reduced by 44% in comparison to control embryos (Zhang *et al.*, 2010; Ertzeid and Storeng, 2001). Our results surprisingly indicated however, that the human

gonadotrophin preparations, despite having improved embryo development compared to PMSG, had reduced numbers of live pups per embryo transferred (mean live offspring rate). The cause for this is uncertain, but one potential theory is blastocysts at a more advanced developmental stage, observed more so following treatment with human gonadotrophins, may miss the implantation window in the mouse, but further research will be required to confirm this.

6.4.2 Ovarian stimulation alters post natal growth

Previous studies reporting the effect of ovarian stimulation on offspring have focused primarily on the foetal stages of development and not monitored growth abnormalities post partum. It has previously been reported that Day 18 mouse foetuses following treatment with PMSG showed a 9% reduction in foetal weight compared to un-stimulated blastocysts which were transferred to the adjacent uterine horn of the un-stimulated surrogate (Zhang *et al.*, 2010). This discrepancy was exacerbated following spontaneous pregnancy to rates as high as 45-48%, supporting the theory that ovarian stimulation also has effects on the endometrium, in addition to the embryo (Ertzeid *et al.*, 1993; Ertzeid and Storeng, 1992). The transfer of embryos to a superovulated surrogate, resulted in a 72% decline in implantation rate compared to transfer to females mated with vasectomised males (Ertzeid and Storeng, 2001), therefore in our study, blastocyst transfer was only performed on females mated to sterile males to specifically assess the influence of ovarian stimulation during folliculogenesis on oocyte quality.

Foetal weight after ovarian stimulation has consistently been reported to be lower than un-stimulated controls (Ertzeid *et al.*, 1993; Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). Although foetal weight was not directly assessed in our study, post natal overgrowth, termed "catch up" growth has been suggested to be an indicator of IUGR (Ceelen *et al.*, 2009). There was an effect of ovarian stimulation on post natal total body weight for the human gonadotrophin preparations when

compared to the transfer of un-stimulated blastocysts. In comparison to *in vivo* controls, all embryo transfers resulted in offspring with higher body mass, including gonadotrophin treated and unstimulated blastocysts. This may indicate that abnormal post natal growth may be due to *in vitro* culture and/or the embryo transfer procedure itself, which potentially could be further confounded by ovarian stimulation.

Day 17 neonates derived following ovarian stimulation with PMSG and 0.5IU hMG had reduced relative heart weight compared to *in vivo* counterparts, indicating superovulation combined with *in vitro* culture and embryo transfer may affect development of the cardio-vascular system. Low birth weight and the association with coronary heart disease and hyper-tension in later life has been previously indicated, although not well defined (Ceelen *et al.*, 2008; Barker, 2002). Low birth weight has been suggested to be associated with ovarian stimulation in humans (Pelinck *et al.*, 2010), however other have not found this trend (Griesinger *et al.*, 2008). Comparison of clinical data is confounded by patient demographic, different fertility aetiologies and duration of infertility which some authors argue may predispose sub-fertile patients to the reported adverse peri-natal risks, regardless of whether ART treatment was undertaken (Kondapalli and Perales-Puchalt, 2013). The evidence of reduced heart mass relative to total body weight only in neonates from ovarian stimulated regimes in fertile mice, offers some support to the theory that ovarian stimulation may be the putative origin of cardiovascular disease in early childhood (Yeung and Druschel, 2013).

Our study also showed ovarian stimulation caused a reduction in relative lung mass. Some authors have suggested infants conceived through ART have a higher incidence of childhood illness requiring healthcare utilisation, in particular for respiratory complaints (Koivurova *et al.*, 2003). The smaller lung mass in progeny from blastocysts transferred following superovulation may lead to an alteration in lung function in adulthood, however further histological and epidemiological studies are

required to confirm this theory. It is important to note that correlations between childhood illnesses and ovarian stimulation is not found by all authors (Bonduelle *et al.*, 2005), however the results of this study does indicate there may be some underlying developmental abnormalities for the adverse neo-natal risks some authors have attributed to ART. Disproportionate foetal and organ growth, in addition to large offspring is well studied in ruminants (see section 1.8.2.2 on LOS). The results of this study may show some mild phenotypes of this condition in mice, as a result of the ovarian stimulation regimes or the procedures themselves, as discussed in the next section.

6.4.3 ART alters pregnancy rates and post natal growth

Previous transfers of blastocysts treated with PMSG to un-stimulated pseudo-pregnant mice reported implantation rates of 50-67.5% (Fossum *et al.*, 1989; Zhang *et al.*, 2010; Van der Auwera and D'Hooghe, 2001), much lower than the 19% live offspring rate reported in our study. However, these research groups did not perform *in vitro* culture, but rather collected blastocysts and transferred them directly to the pseudo-pregnant recipients. The transfer of pronuclear embryos immediately after collection resulted in a 63% implantation rate on day 15, compared to only 47% when embryos were cultured for 29 hours (Van der Auwera *et al.*, 1999). To the best of our knowledge, only one other research group performed blastocyst culture followed by embryo transfer and reported an implantation rate on day 18 of only 12% for embryos treated with PMSG and 25% for un-stimulated control embryos (Ertzeid and Storeng, 2001), comparable to our live offspring data. These results indicate that *in vitro* culture may be a confounding factor for post-natal growth abnormalities following ART. *In vitro* culture and embryo transfer alone in this study caused additional adverse effects including larger kidneys and heavier spleens in both stimulated and un-stimulated treatment groups. The observation that unstimulated controls had some statistically different organ weight compositions compared to *in vivo* controls supports this hypothesis.

6.4.4 Conclusion

The results of this study suggest that superovulation may alter development *in vivo* which may result in long term consequences for offspring born following ART procedures. *In vitro* culture resulted in adverse effects on offspring growth and this was shown to be further exacerbated by ovarian stimulation. This may provide a possible mechanism for the "Developmental Origins of Health and Disease" hypothesis and highlights the paramount importance of monitoring the long term consequences of superovulation in ART progeny (Barker, 1995).

CHAPTER 7: THE EFFECT OF OVARIAN STIMULATION ON THE ABILITY OF THE EMBRYO TO WITHSTAND IN VITRO MANIPULATIONS

7.1 INTRODUCTION

The use of transgenic mice to study human disease models accounts for a third of all experimental animal usage. The generation of transgenic animals requires micro-injection of either DNA or a gene transfer construct. Transgenic zygote production rates however are suboptimal and rely heavily on the use of superovulated animals, commonly using PMSG. It is therefore essential that the quality of embryos retrieved after ovarian stimulation is high in order to survive both *in vitro* culture and micro-manipulation stresses (Brooke *et al.*, 2007). Our results like others in the literature show ovarian stimulation with PMSG reduces the proportion of blastocysts and delays development both *in vitro* (Ertzeid and Storeng, 1992; Van der Auwera and D'Hooghe, 2001) and *in vivo* (Ertzeid and Storeng, 2001). Comparisons of human gonadotrophin superovulation regimes in mice are conflicting, due to the various strains and age of mice used, combined with the different doses and timing of gonadotrophin injections (Brooke *et al.*, 2007; Edirisinghe *et al.*, 1986; Calongos *et al.*, 2008; Muñoz *et al.*, 1994). Different culture media used by research groups may also influence their *in vitro* embryo development data. There is no direct comparison in the literature of the most clinically used human gonadotrophin preparations in a single mouse strain, which may be more suitable for transgenic applications.

Pre-implantation genetic diagnosis (PGD) for couples at risk of transmitting an inheritable chromosomal or genetic disorder (Handyside *et al.*, 1990), is gaining in popularity, with 383 cycles performed in 2010 (Harper *et al.*, 2012). It permits detection of affected embryos prior to transfer back to the uterus. Aneuploidy screening (detection of abnormal

chromosome number) termed pre-implantation genetic screening (PGS) is a sub-type of PGD used for females of advanced maternal age or couples that have had repeated IVF failure or recurrent miscarriages previously (Gianaroli *et al.*, 2005). As 40% of transferred embryos after IVF demonstrate some form of chromosomal abnormality it is thus hypothesised that genetic screening of embryos will mainly increase the pregnancy rate in this selected group of patients (Kahraman *et al.*, 2004). Currently there is insufficient data to either confirm or dispute these claims (Twisk *et al.*, 2006). In both PGD and PGS, controlled ovarian stimulation is employed to increase the number of embryos available to be tested. How stimulation with different regimes affects the ability of embryos to recover following a biopsy has not previously been assessed.

As more cells can be successfully retrieved following blastocyst biopsy, more clinics are adopting this technique to test for monogenic heritable diseases (Chang *et al.*, 2013). The 1.48μm diode non contact infra-red laser has previously been shown, when utilised to drill the zona pellucida of oocytes, to have no adverse effects on the ability of subsequently fertilised mouse embryos to develop (Germond *et al.*, 1995). Implantation rates following use of the laser was also unaffected, resulting in comparable offspring numbers compared to oocytes that were undrilled (Germond *et al.*, 1996). The laser, which works by a local thermal effect through the adsorption of the energy by water and/or macromolecules leading to a confined thermolysis (Rink *et al.*, 1996), has also been shown to be safe to drill human embryos for cleavage stage biopsy (Boada *et al.*, 1998). Accumulating clinical evidence now suggests its use for blastocyst biopsy has no adverse effect on the embryo, implantation or development to term (Kokkali *et al.*, 2005; McArthur *et al.*, 2008; McArthur *et al.*, 2005). There have been no in depth studies into how the blastocyst responds to the biopsy procedure in a research setting.

The objective of this experiment was to examine the efficacy of common human gonadotrophin preparations with different half lives and LH activity

in mice (hMG and Pergoveris) on the ability of blastocysts to respond to stress in terms of micromanipulation. The endpoints measured in this experiment were blastocyst grade pre and post biopsy and the effect of biopsy on recovery and imprinted gene expression, which are hypersensitive to disruptions during micromanipulation.

7.2 METHODS OVERVIEW

Mice were randomly allocated to the various treatment groups as described in chapter 2 section 2.1:

Table 7.1: Number of mice in each treatment group

Treatment	Number of technical repeats (n)	Total number of mice (n)
Un-stimulated	18	72
5IU PMSG	12	49
0.5IU hMG	9	36
5IU hMG	6	23
0.5IU Pergoveris	9	36
5IU Pergoveris	10	41

After blastocyst grading, those hatching at the site of the ICM were triple stained (Results section 4.3.2.8), whereas those hatching away from the vicinity of the ICM underwent trophectoderm biopsy (as detailed in chapter 2 section 2.4.2). Hatched blastocysts were not biopsied due to difficulty holding them stationary without the zona pellucida. Using Garner and Schoolcraft's blastocyst grading system (section 2.3.2.2) these are

all blastocysts graded between 5AA and 5CC. Un-stimulated (n=12), PMSG (n=9), 0.5IU hMG (n=11), 5IU hMG (n=11), 0.5IU Pergoveris (n=10) and 5IU Pergoveris (n=12) biopsied samples were chosen at random so as not to introduce bias. They were designated for polymerase chain reaction (PCR) for gene expression analysis (Section 2.4.3).

After re-incubation for 3 hours, blastocysts were re-graded and those with sufficient hatching to permit a second biopsy were re-biopsied and re-analysed for imprinted gene expression. Only the natural (n=11), PMSG (n=9) and 0.5IU hMG (n=11) groups were re-biopsied and analysed for *H19, IGF2* and *IGF2r* gene expression, as detailed in section 2.4.3. Those that were not, were triple stained to analyse any effects of the biopsy procedure on apoptosis rates. After the second biopsy, blastocysts were also triple stained to check for viability. Non-biopsied and biopsied blastocysts from the natural, PMSG and 0.5IU hMG groups (n=20 per group) underwent electron microscopy using an environmental scanning electron micrograph (eSEM) in order to view the response to trophectoderm biopsy under high magnification and resolution. The experimental pathways in this chapter are schematically represented in Figure 7.1.

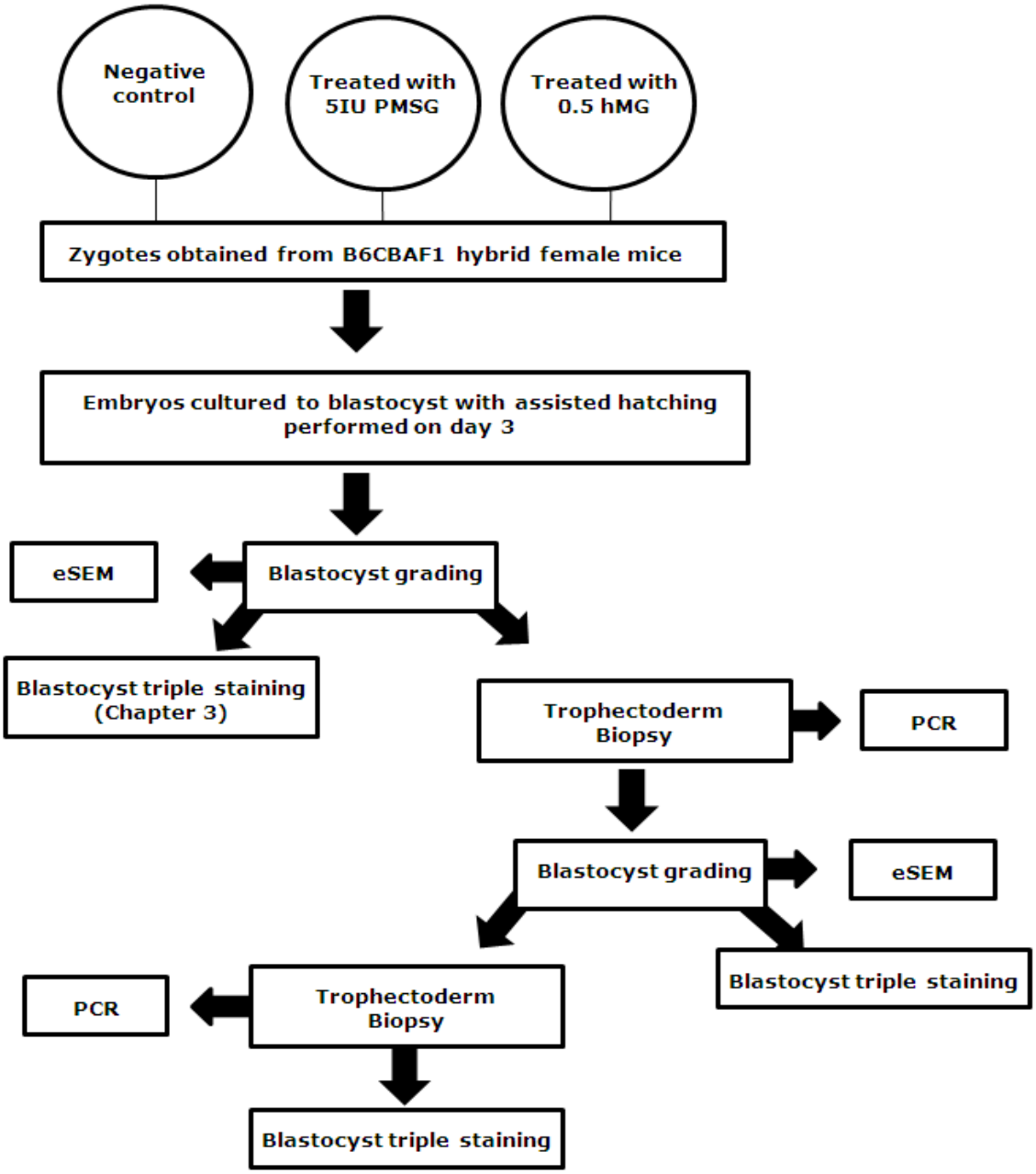

Figure 7.1: Flow chart summarising the experimental design used in this chapter.

7.2.1 Environmental SEM

Environmental scanning electron micrography (eSEM) was used to analyse the surface structure of blastocysts pre and post trophectoderm biopsy to see the morphological consequences of the laser at high power and resolution. This was used over conventional SEM to permit the observation of the blastocysts in their natural state without the need for de-hydrating, critical point drying and coating which may alter the shape of the cells and introduce artefacts.

7.2.1.1 Sample fixation and preparation

Non-biopsied and biopsied blastocysts were allowed to recover for 3 hours, and subsequently were fixed in 4% PFA in glass embryo dishes overnight to prevent damage to the blastocysts cell integrity from the chamber conditions during the initial de-hydration step. On the following day, blastocysts were transferred to PBS drops under oil and the zona pellucida was removed using the Integra micromanipulators. Whilst the blastocyst was held stationary with a 9-17μm thick walled blunt holding pipette on the zona pellucida, PBS media was gently expelled inside the cavity using a Swemed pipette. Eventually the blastocyst would flow out of its protein cocoon with minimal disruption to its surface structures.

7.2.1.2 eSEM

Fixed blastocysts were washed several times in distilled water to avoid salt contamination from the PBS interfering with the eSEM. Blastocysts were individually transferred using a Swemed to the centre of a 5 micron nuclepore poly-carbonate filter (Nuclepore Track Etch membrane, Whatman, Kent, UK) previously mounted onto a stub. Two extra droplets of water were added to the centre of the filter to maintain hydration during wet mode imaging of the blastocyst. The stub was then transferred to the chamber of the eSEM (Gaseous secondary electron imagery XL30 eSEM-FEG-FEI) and the conditions set at 3°C for the cooling stage, 2X flood, -10kV, WD 8.1-8.6, 3.5-4 Torr, corresponding to 72-79% relative

humidity. This allowed gradual evaporation of the surface layer of liquid from the cells and the blastocyst sample images were recorded.

7.2.2 Statistical analyses

Data was tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests using SPSS version 16 (IBM Software Services, Hampshire, UK). Statistical relevance was set at $P<0.05$ for all analyses. Statistical analyses of the blastocyst triple staining data were performed on SPSS for cell count data and also on Graph Pad prism 6 using chi squared test for associations for the analysis of dichotomous proportional data. All continuous cell count data was normally distributed and analysed by ANOVA. Gene expression was not normally distributed and could not be successfully transformed by logarithm or square root so was graphically represented by a box plot and analysed using the non-parametric Kruskal-Wallis test on SPSS. Any sample with an 18s Ct value above 26 was omitted due to lack of biological significance. Categorical blastocyst grade classifications were assessed using chi squared test on Graph Pad.

7.3 RESULTS

7.3.1 Evidence of stress

7.3.1.1 Blastocyst quality prior to biopsy

Figure 7.2 gives the proportion of blastocysts within each grade category that underwent trophectoderm biopsy in the Natural (n=105), PMSG (n=143), 0.5IU hMG (n=112), 5IU hMG (n=21), 0.5IU Pergoveris (n=21) and 5IU Pergoveris (n=42) treatment groups. Treatment with PMSG, 0.5IU hMG and 5IU hMG resulted in less top quality 5AA grade blastocysts compared to naturally derived controls and a higher proportion of lower graded 5CC blastocysts.

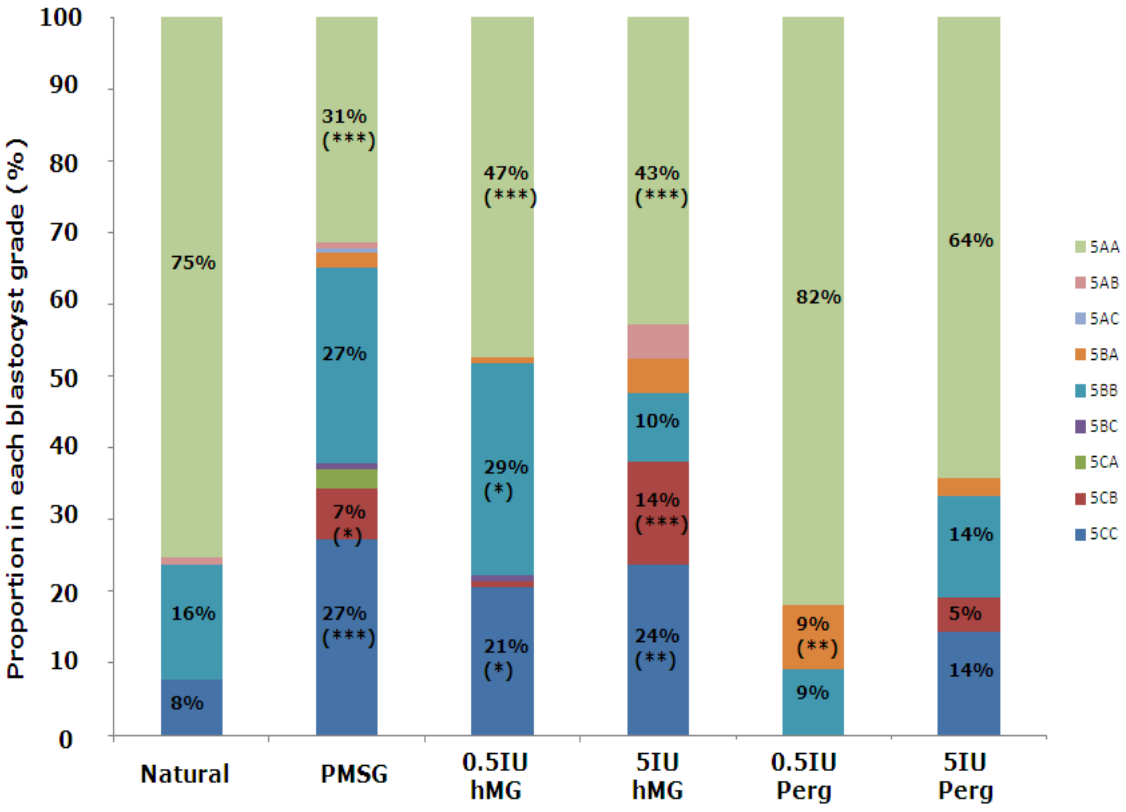

Figure 7.2: Graph showing the proportion of blastocyst grades prior to biopsy in the treatment groups. (* P<0.05, ** P<0.01 and *** P<0.001 compared to natural as assessed by chi squared test).

7.3.2 Response to stress

7.3.2.1 Blastocyst survival

Biopsied blastocysts derived from unstimulated controls had a 93±3% re-expansion rate 3 hours after re-incubation (Figure 7.3). Treatment with PMSG however resulted in significantly less blastocysts recovering from the biopsy procedure (P<0.01). The 0.5IU hMG, 5IU hMG, 0.5IU Pergoveris and 5IU Pergoveris treatment groups had comparable recovery rates to the unstimulated control (P>0.05).

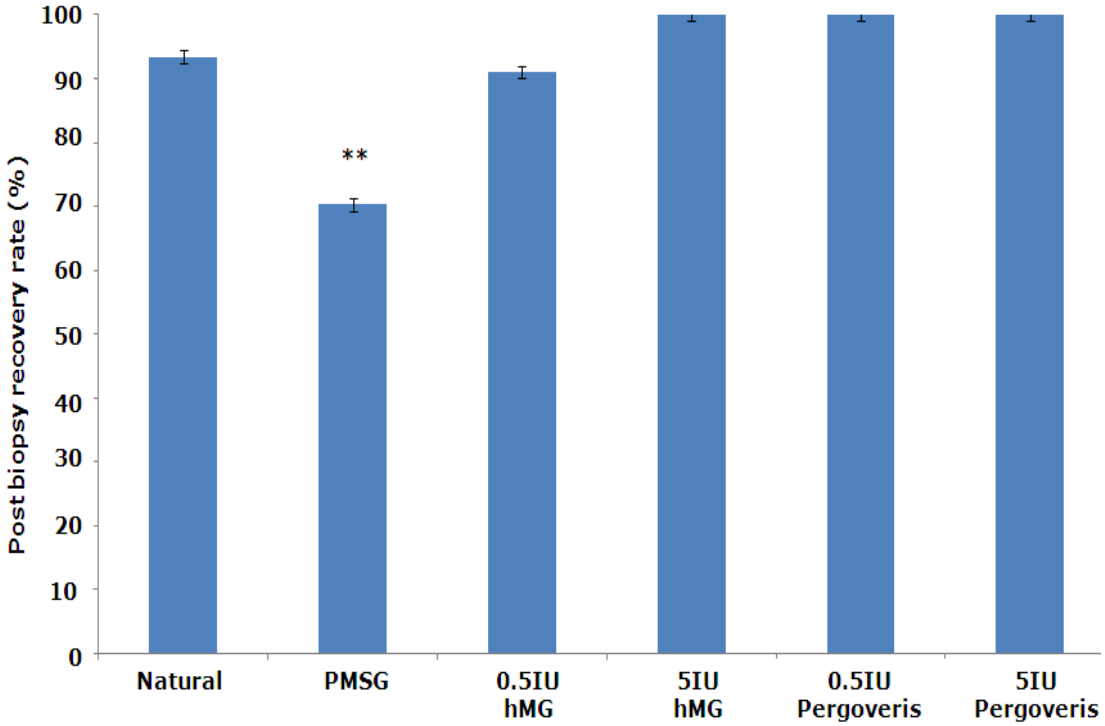

Figure 7.3: Mean ± SEM re-expansion rates 3 hours post biopsy for the natural (n=72), PMSG (n=49), 0.5IU hMG (n=36), 5IU hMG (n=23), 0.5IU Pergoveris (n=36) and 5IU Pergoveris (n=41) treatment groups. (** P<0.01 compared to natural as assessed by chi squared test).

7.3.2.2 Post biopsy blastocyst grade

The proportion of blastocysts re-graded 3BB or better 3 hours post biopsy in the natural group was 76%. This was comparable to those treated with 5IU hMG where 82% were reclassified in this morphological range. Biopsied blastocysts after treatment with PMSG however only had 51% good quality blastocysts ($P<0.001$). In contrast to this, the 0.5IU hMG and both Pergoveris treatment groups had improved blastocyst recovery after the biopsy procedure (91% $P<0.01$ and 100% $P<0.001$ respectively). The majority of the biopsied blastocysts from natural controls were re-graded as 5AA and 4AA (Figure 7.4). However, there were significantly more blastocysts with poor grades following treatment with PMSG ($P<0.001$), with nearly half of the group being re-graded as 3CC or 2CC due to limited re-expansion of the blastocoel cavity.

The 0.5IU hMG group had better recovery rates ($P<0.01$) with most biopsied blastocysts re-graded as 5AA. Treatment with 5IU hMG has resulted in comparable re-expansion rates to the natural group with most blastocysts 3 hours post biopsy graded 5BB and 4BB ($P>0.05$). The Pergoveris treatment groups recovered faster than the natural group post biopsy ($P<0.001$) with the majority of blastocysts graded 5AA and surprisingly these stimulation groups were the only groups to have any blastocysts to continue to hatch fully within the 3 hour post biopsy window (Videos of re-expansion can be viewed on the CD Rom accompanying this thesis). Representative examples of blastocyst appearance 3 hours post biopsy for the different treatment groups are shown in Figure 7.5.

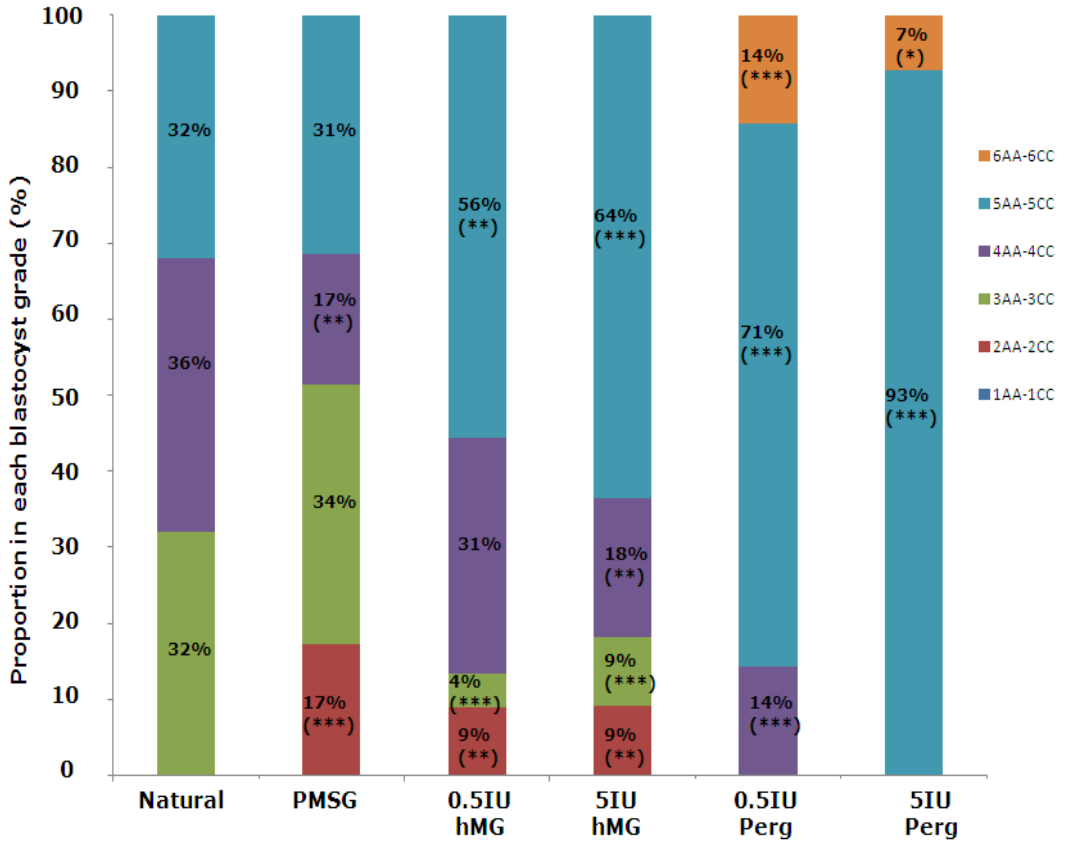

Figure 7.4: Blastocyst grade of embryos 3 hours post biopsy for the treatment groups. (* P<0.05, ** P<0.01, *** P<0.001 compared to natural as assessed by chi squared test).

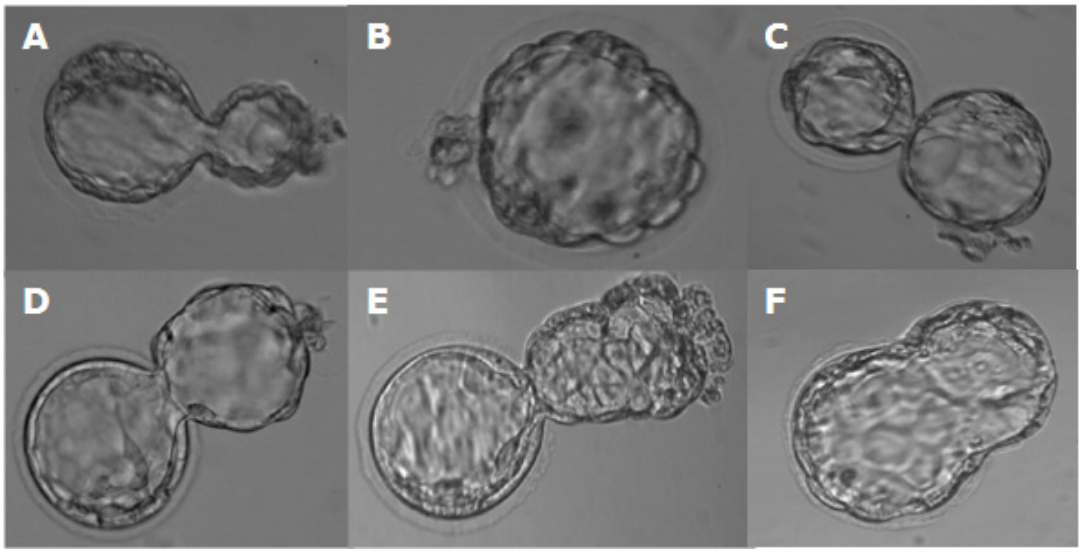

Figure 7.5: Representative images of re-expanded blastocysts 3 hours post biopsy for the **(A)** Natural, **(B)** PMSG, **(C)** 0.5IU hMG, **(D)** 5IU hMG, **(E)** 0.5IU Pergoveris and **(F)** 5IU Pergoveris groups.

7.3.2.3 Re-biopsy gene expression profiles

7.3.2.3.1 Unstimulated blastocysts gene expression

The relative median fold difference of *H19* gene expression was similar for the first and second biopsied samples for blastocysts derived from naturally cycling controls (P>0.05, Figure 7.6). *IGF2* expression showed a trend for diminished expression in the second biopsy, with two thirds the amount of mRNA transcripts being detected compared to the first biopsy (P=0.051). The relative fold difference of *IGF2r* was comparable between the two biopsies (P>0.05).

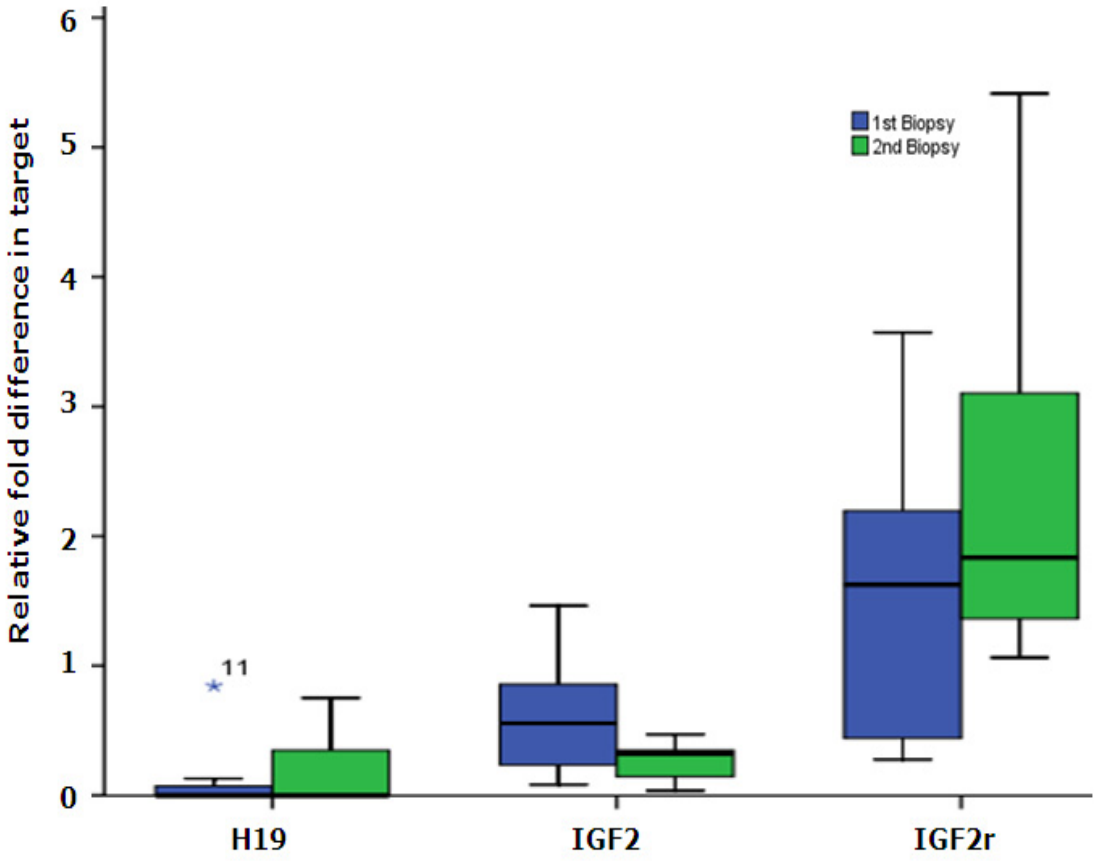

Figure 7.6: Median relative expression of *H19, IGF2* and *IGF2r* in the natural treatment group (n=11) within the 1st and 2nd biopsy. Error bars depict the inter-quartile range.

7.3.2.3.2 PMSG treatment group gene expression

The relative median fold difference of *H19* gene expression was comparable for the first and second biopsied samples in the PMSG treated group (P>0.05, Figure 7.7). *IGF2* expression was ten-fold lower in the second biopsy sample compared to the initial biopsy, however this trend was not significant (P>0.05). The relative fold difference of *IGF2r* was comparable between the two biopsies (P>0.05).

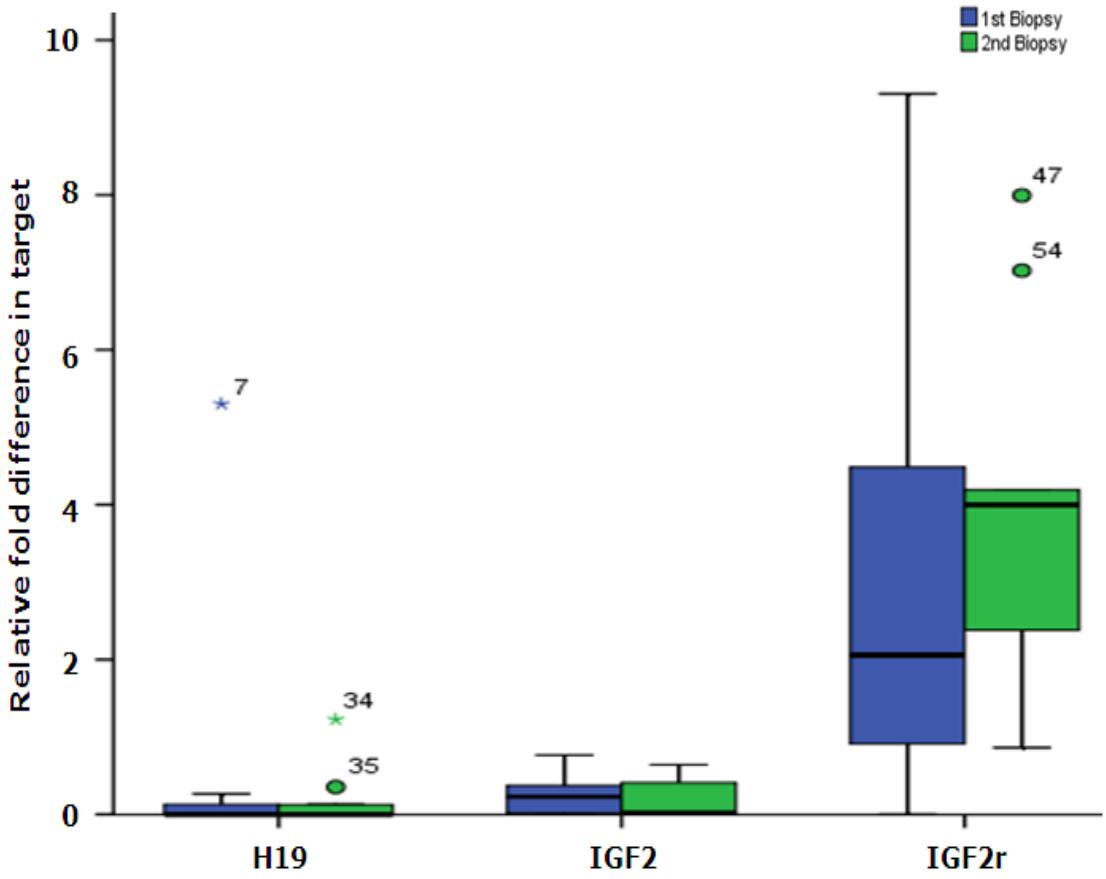

Figure 7.7: Median relative expression of *H19, IGF2* and *IGF2r* in the PMSG treatment group (n=9) within the 1st and 2nd biopsy. Error bars depict the inter-quartile range.

7.3.2.3.3 0.5IU hMG stimulated blastocyst gene expression

The relative median fold difference of *H19* gene expression was similar for the first and second biopsied samples in the 0.5IU hMG group (P>0.05, Figure 7.8). *IGF2* and *IGF2r* expression was also not significantly different between the biopsy samples (P>0.05). The 0.5IU hMG stimulation group had the most analogous results between the initial and re-biopsied cell samples.

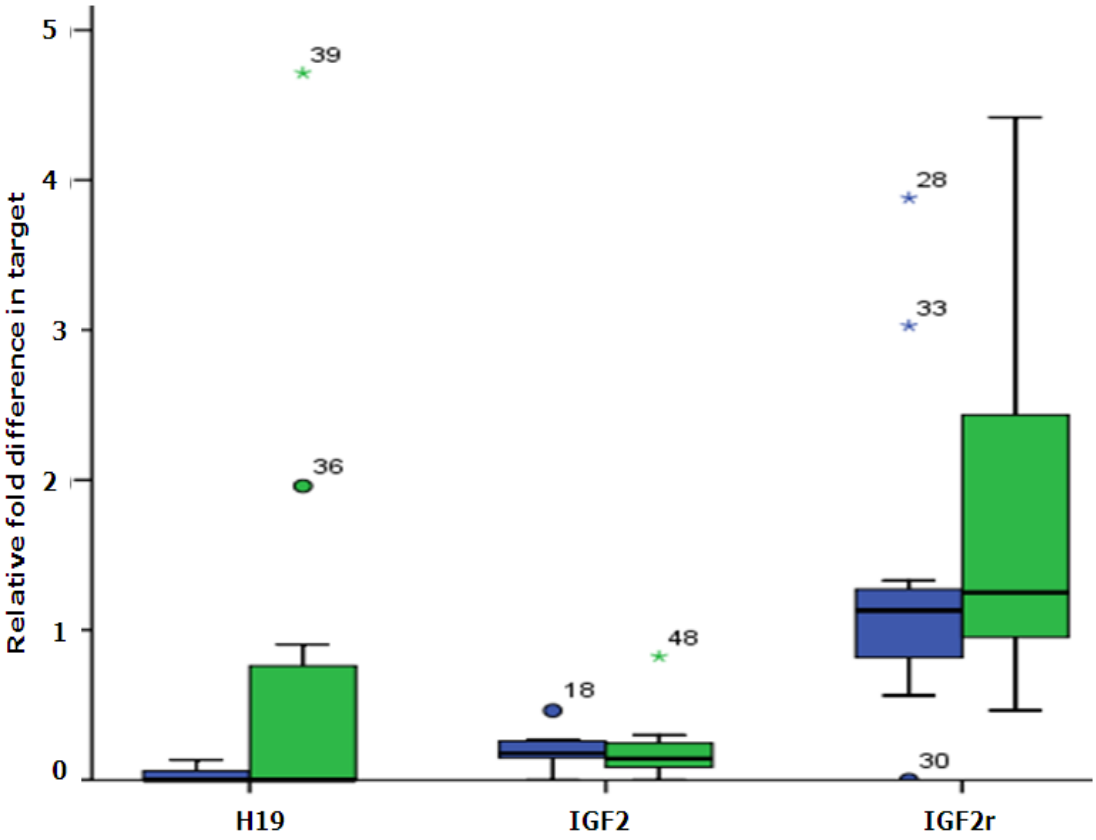

Figure 7.8: Median relative expression of *H19, IGF2* and *IGF2r* in the 0.5IU hMG treatment group (n=11) within the 1st and 2nd biopsy. Error bars depict the inter-quartile range.

7.3.3 Morphological response

7.3.3.1 Biopsied blastocysts triple staining

The findings of the non-biopsied blastocysts triple staining count have previously been presented in section 4.3.2.8, but have also been incorporated in Table 7.2 to allow comparison of the data. The total cell number (TCN) of biopsied blastocysts for the natural, PMSG and 0.5IU hMG treatment groups were significantly reduced compared to non-biopsied counterparts ($P<0.01$) but re-biopsied blastocysts following treatment with PMSG were equivalent to non-biopsied controls ($P>0.05$). Non-biopsied and biopsied blastocysts from the 5IU hMG group were comparable in terms of cell number ($P>0.05$) and the 0.5IU Pergoveris group had more cell numbers in the biopsied blastocysts than non-biopsied, although this trend was not statistically significant ($P>0.05$). Conversely, the TCN of biopsied blastocysts following treatment with 5IU Pergoveris was significantly reduced after the biopsy procedure ($P<0.001$). Overall as would be expected, blastocyst biopsy reduced the cell number of blastocysts, regardless of treatment group.

The ICM and TE proportion was unaffected by the initial biopsy procedure ($P>0.05$). The TE proportion was reduced after the biopsy, though this trend was not significant ($P>0.05$). Re-biopsies of blastocysts from the PMSG treated group had significantly less TE proportion compared to non-biopsied and biopsied blastocysts ($P<0.01$) whereas re-biopsied 0.5IU hMG treated blastocysts had a comparable TE proportion ($P>0.05$). There was no significant elevation of apoptosis in any of the treatment groups for the initial biopsy procedure ($P>0.05$), however biopsied blastocysts following treatment with 0.5IU Pergoveris displayed a trend for higher prevalence of apoptosis compared to non-biopsied blastocysts of the same group ($P=0.0513$). Re-biopsied blastocysts from naturally cycling controls had comparable apoptosis rates to their non-biopsied and biopsied counterparts ($P>0.05$). In converse, PMSG treated re-biopsied blastocysts had a significant increase in proportion of apoptosis compared with non-biopsied and biopsied ($P<0.05$). The re-biopsied blastocysts following

treatment with 0.5IU hMG also showed a trend for increased proportion of apoptosis compared to non-biopsied counterparts (P=0.051). In summary, ovarian stimulation did not alter the TCN, ICM, TE or apoptosis counts following biopsy.

Table 7.2: Summary of fluorescent blastocyst triple staining count for non-biopsied, biopsied and re-biopsied. (* P=<0.05, ** P=<0.01, *** P=<0.001 for biopsied and re-biopsied compared to non-biopsied in the same treatment) (a P=<0.05, b P=<0.01 for re-biopsied compared to biopsied as assessed by ANOVA and chi squared test).

Treatment	Total Cell numbers (TCN)	ICM proportion	TE proportion	Proportion of apoptotic cells (%)
Non-Biopsied				
Natural (n=72)	83±6	46±2	54±2	5±1
PMSG (n=49)	67±5	39±1	61±1	9±1
0.5IU hMG (n=36)	82±5	45±2	55±2	5±1
5IU hMG (n=23)	101±10	39±3	61±3	9±1
0.5IU Pergoveris (n=36)	83±9	40±2	60±2	5±1
5IU Pergoveris (n=41)	112±5	41±1	59±1	3±1
Biopsied				
Natural (n=20)	49±3(**)	48±1	52±1	7±1
PMSG (n=26)	40±4(**)	42±1	58±1	9±2
0.5IU hMG (n=44)	55±3(***)	54±1	46±1	7±1
5IU hMG (n=18)	99±9	43±1	57±1	12±2
0.5IU Pergoveris (n=16)	89±10	40±1	60±1	14±2
5IU Pergoveris (n=50)	86±5(***)	45±1	55±1	8±1
Re-biopsied				
Natural (n=8)	41±4 (**)	60±1	40±1	8±1
PMSG (n=10)	41±9	60±1	40±1(**,b)	18±3 (*,a)
0.5IU hMG (n=22)	49±6 (***)	51±1	49±1	14±4

Representative fluorescent triple stained biopsied and re-biopsied blastocysts can be found in Figure 7.9 for the unstimulated, PMSG, 0.5IU hMG, 5IU hMG, 0.5IU Pergoveris and 5IU Pergoveris treatment groups. Unlike non-biopsied blastocysts where apoptotic cells were randomly dispersed throughout the embryo (See Figure 4.14 in section 4.3.2.8), the location of the apoptotic cells within the biopsied and re-biopsied blastocysts were located mainly at the site of laser contact and where the embryos were held stationary by mild suction.

7.3.3.2 SEM of blastocysts

Both natural cycling controls and 0.5IU hMG treated blastocysts have clearly discernible cells with their structures resembling cobble-stones. Blastocysts following treatment with PMSG however appear smooth, with individual cells harder to distinguish. Post biopsy, the area exposed to the laser has a different morphology compared to the rest of the blastocyst at 1000X magnification. At 4000X magnification it is clearer that this region of the blastocyst has more cells in tighter apposition. Cells within the rest of the embryo have a few noticeable perforations and appear transparent, whereas those at the site of biopsy look less porous and more opaque. Upon closer examination at 8000X magnification, the cells which were pulled apart for the final stage of the biopsy have, in some cases formed fringed structures which have sealed (Figure 7.10D). Others such as those shown in Figure 7.10L have a rough surface, which appear to have cells adhered to each other with what looks to be a protein coat on top.

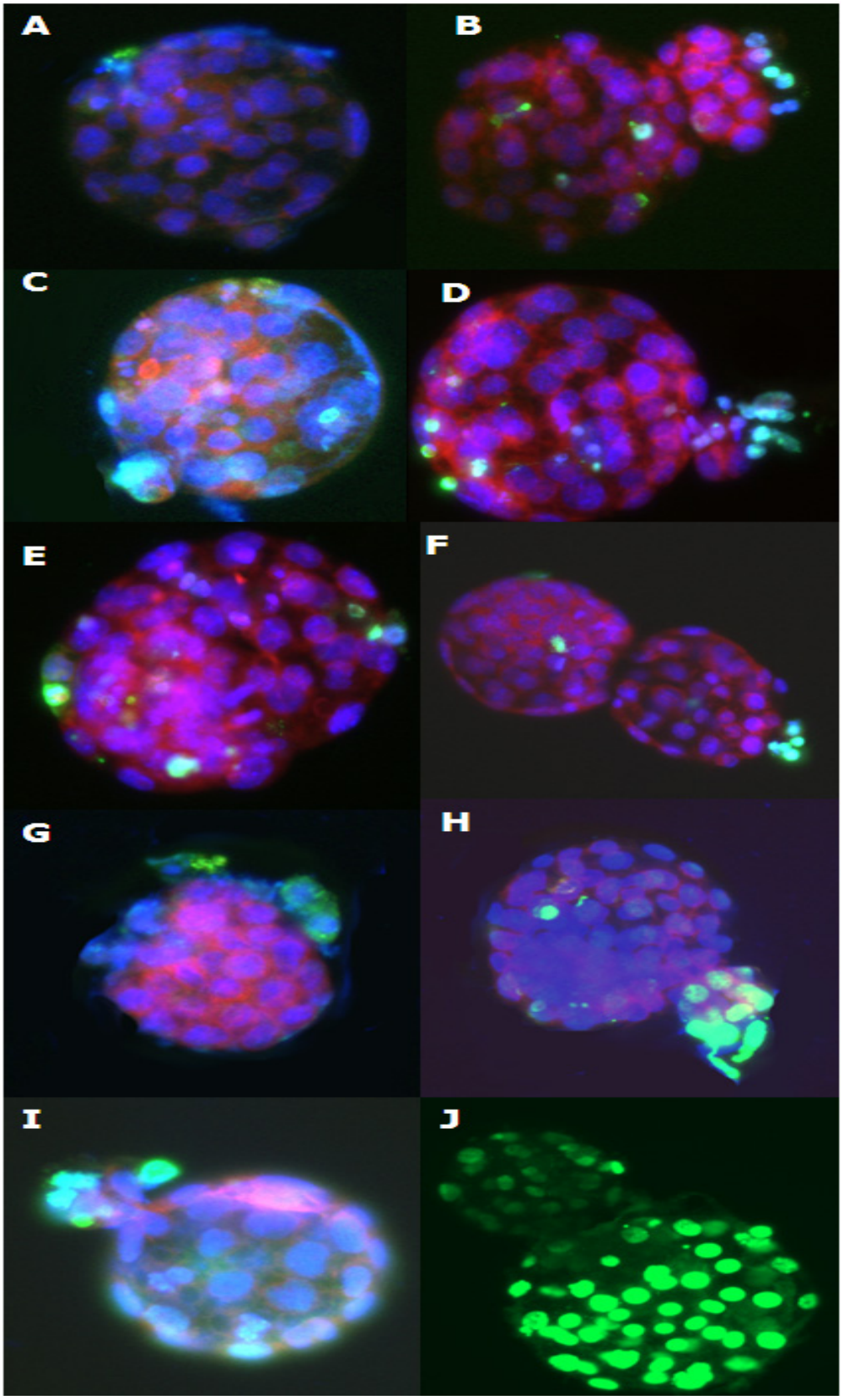

Figure 7.9: Representative examples of biopsied **(A)** natural, **(B)** PMSG, **(C)** 0.5IU hMG, **(D)** 5IU hMG, **(E)** 0.5IU Pergoveris and **(F)** 5IU Pergoveris blastocysts and re-biopsied **(G)** natural, **(H)** PMSG and **(I)** 0.5IU hMG blastocysts and a **(J)** positive tunel control.

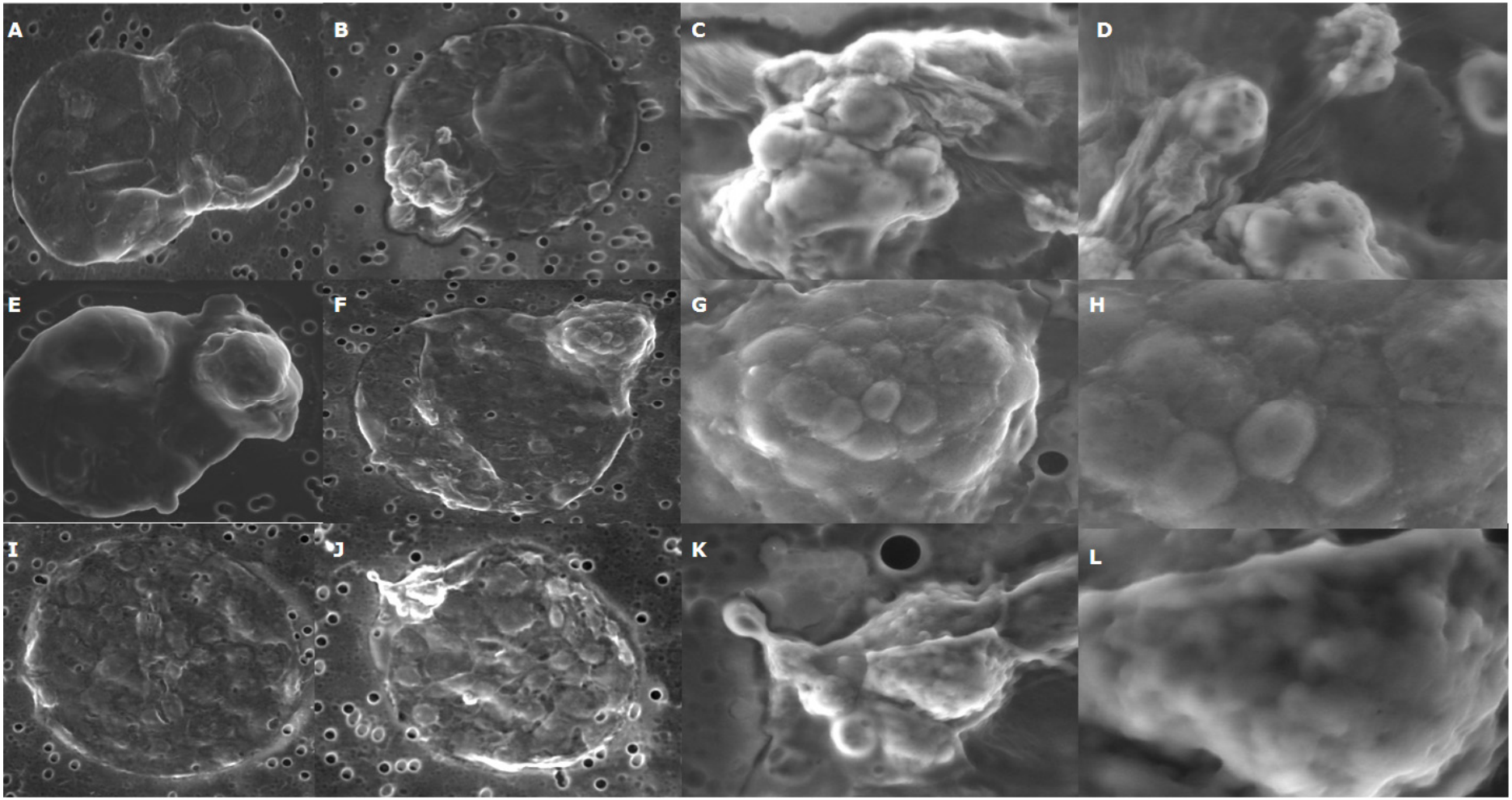

Figure 7.10: Representative eSEM images of non-biopsied blastocysts (1000X), biopsied blastocysts (1000X) and biopsied area at 4000X and 8000X magnification for blastocysts derived from **(A-D)** natural, **(E-H)** PMSG and **(I-L)** 0.5IU hMG blastocysts.

7.4 DISCUSSION

In this study it was hypothesised that based on the previously reported detrimental embryo development following treatment with PMSG (Chapter 3), blastocysts treated with human gonadotrophins will be less effected by stress. The results obtained suggest that trophectoderm biopsy on PMSG treated blastocysts, results in reduced recovery rates and poor blastocyst quality. Blastocysts following treatment with human gonadotrophins, on the other hand, had better recovery rates and higher graded blastocysts post biopsy. Ovarian stimulation can affect the ability of embryos to respond to stress but these effects were not apparent with the human preparations. This also has implications for the widespread use of PMSG to produce embryos for research, such as for transgenic studies.

7.4.1 Ovarian stimulation alters blastocyst response to stress

Blastocysts following treatment with PMSG were more sensitive to *in vitro* biopsy manipulations than the other treatment groups and un-stimulated control, based on their poor recovery following trophectoderm biopsy. Although the initial morphological blastocyst grades of embryos from the PMSG treatment group were poor, therefore potentially already reducing the chance of survival, alternative ovarian stimulation groups with comparable grades of blastocysts were not impaired, implying that it is the use of PMSG which is responsible for poor survival following biopsy. Furthermore, although PMSG appeared to have a large number of cells removed at the time of biopsy, as indicated by the TCN data (Table 7.2), which may have increased the detrimental impact of the biopsy, other treatments had analogous cell number declines following biopsy, indicating it may be the stimulation regime responsible for poor survival. In addition to poor recovery rates, re-grading of the blastocysts indicated half of them were less than 3BB, the grade above which has been shown to have better implantation potential and improved pregnancy rates (Teranishi *et al.*, 2009). This implies that after biopsy, blastocysts following treatment with PMSG may not recover sufficiently to instigate a

pregnancy. This is an important finding, as PMSG is commonly used to produce numerous embryos for transgenic studies. This negative effect was not observed following ovarian stimulation with human gonadotrophins, suggesting stimulation of mice with these preparations may be more suitable for transgenic applications and in addition provides some reassurance of the safety of this procedure on human embryos following ovarian stimulation with human gonadotrophins.

Treatment with Pergoveris was previously shown to increase the developmental speed of embryos resulting in more hatched blastocysts after *in vitro* culture (Chapter 3). Recovery rates following trophectoderm biopsy were also faster than other groups with over 90% of blastocysts 3 hours post biopsy graded 3BB or better. Significantly this group was the only treatment that had fully hatched blastocysts post biopsy, indicating blastocysts following treatment with 5IU Pergoveris are more capable of continuing development following micromanipulation stresses. This observation should be further tested with other stressors, such as cell injections because embryos which are more resilient to environmental stress may be more suitable for transgenic applications than PMSG retrieved embryos which in our study we showed to be the most sensitive to external influences.

7.4.2 Safety of trophectoderm biopsy

The trophectoderm biopsy work carried out indicated the micro-manipulation procedure had no adverse effects on blastocyst recovery, apoptosis or imprinted gene expression providing evidence for the safety of the 1.48μm diode non contact infra-red laser used in this study. Our study demonstrates the safety of trophectoderm biopsy in a research setting, based on large numbers of mouse blastocysts following ovarian stimulation with multiple regimes which complements the few reports of clinical data in the literature. Furthermore our results also showed re-biopsy of blastocysts did not have any effects on apoptosis except for

those following treatment with PMSG, which do not recover well after the initial biopsy. This observation suggests that it is safe to re-biopsy embryos if a null result is received first time, a practice which is deemed acceptable by the ESHRE PGD consortium (Harper *et al.*, 2012). This has previously only been reported when a blastocyst was re-biopsied after no result was obtained following a cleavage stage biopsy (Wininger *et al.*, 2011). We show it may be possible to safely re-biopsy a blastocyst staged embryo with a failed result, which depending on the speed of the clinics output would enable embryo transfer the same day, the following day (Day 6 transfer) or cryo-preservation until a result is received (Chang *et al.*, 2013).

Although apoptosis was not increased by trophectoderm biopsy, the location of these damaged cells was focused at the site of analysis. Environmental scanning electron micrography was used to attempt to visualise the repaired area of the expanded blastocysts. Regardless of treatment, the site of biopsy had a greater number of cells, which is also apparent on fluorescent images and is consistent with the idea that mitosis occurs in response to cell damage. Under high magnification, cells within this region appeared denser and looked to be coated with a substance resembling plaque. This may be the putative mechanism by which the blastocyst recovers from trophectoderm biopsy, sealing the blastocoel cavity, allowing re-expansion. Further analysis into the potential substance of this "embryo scab" would be interesting and perhaps may be similar to wound healing mechanisms documented in adult tissues (Hotary *et al.*, 2002). Alternatively increased tight junction expression may enable sealing of the blastocoel cavity post biopsy, which may explain the lower recovery rate of PMSG blastocysts, which have a smoother appearance than other treatment groups under the eSEM. Gene expression analyses of this region are required to support these speculations.

7.4.3 Biopsy does not alter imprinted gene expression

Gene expression of *IGF2* and *H19* have been shown to be hypersensitive to stresses during *in vitro* culture and been suggested to be useful as a quasi-sensor for epigenetic perturbations (Nan *et al.*, 1998). In our study we re-analysed the second trophectoderm biopsy cell sample to check for any alterations in expression of these imprinted genes which could potentially cause long term consequences in subsequent offspring (Tycko and Morison, 2002). Re-biopsied samples from un-stimulated and superovulated blastocysts showed comparable *H19* and *IGF2* gene expression profiles in comparison to their initial result. Even PMSG treated blastocysts showed similar results despite poorer recovery rates. This observation provides further support for the safety and clinical implementation of this blastocyst biopsy for PGD purposes.

7.5 Conclusion

The results of this study provide evidence that ovarian stimulation with PMSG can affect the response of blastocysts to stress, such as trophectoderm biopsy. The negative effects of the micromanipulation on blastocyst recovery and apoptosis rates were not evident when human gonadotrophins were used. This provides some reassurance for the safety of this procedure when applied to patient treatment in a clinical setting, whilst also indicating superovulation with PMSG may not be optimum for generating embryos for use in transgenic mice applications.

CHAPTER 8: GENERAL DISCUSSION

8.1 Justification and summary of work

The objectives of the work undertaken in this thesis were to assess the impact of different gonadotrophin treatments on oocyte and embryo quality, gene expression and the effects on resulting progeny. The main findings of this thesis is as follows:

1. Ovarian stimulation with PMSG and human gonadotrophins negatively impacts oocyte quality in terms of morphology and developmental potential.
2. Embryo development following ovarian stimulation with PMSG was significantly impeded compared to natural controls. Embryo development following the use of human gonadotrophins was not as severely impacted compared to PMSG, however the quality of resulting embryos appeared to be dependent on the dose, half life and LH content of the preparations used.
3. Although our results showed some alterations in mRNA expression levels of imprinted genes and angiogenic factors, only a subset of genes were analysed. More work is needed with a greater number of repeats at different stages of analysis throughout embryonic development and a wider range of genes assessed (gene array).
4. Ovarian stimulation did alter the growth of progeny post birth in terms of body mass and growth. However, aberrations were apparent in unstimulated controls compared to naturally conceived offspring, suggesting *in vitro* culture and/or the embryo transfer procedure itself may be having an impact.

The research in Chapters 3 and 4 of this thesis assessed whether reports that ovarian stimulation negatively effects *in vitro* embryo development. Ovarian stimulation with PMSG and human gonadotrophins had some negative effects on both embryo development and blastocyst quality,

indicating that ovarian stimulation in general does impact on the developmental competence of oocytes and embryos. This study highlighted two key issues regarding human gonadotrophin preparations which influenced embryo yield and viability. Firstly, preparations composed of recombinant FSH were required at higher concentrations to yield comparable number of embryos compared to preparations with a urinary FSH constitution. Although the more basic FSH iso-forms found in rFSH show greater *in vitro* bioactivity, they have a faster *in vivo* clearance rate compared to the non-basic iso-forms found in uFSH (Vitt *et al.*, 1998). This may potentially explain why lower concentrations of hMG are needed to achieve increased numbers of oocytes as opposed to the low half life recombinant Pergoveris which requires significantly more to achieve a comparable response.

Another significant finding was the apparent negative effect of the long acting LH surrogate, human chorionic gonadotrophin (hCG) within the hMG preparation. Increasing the concentration of hMG led to a decrease in the number of ovulated oocytes and impeded subsequent embryo development and blastocyst quality. This may be due to down regulation of the LH receptor previously shown to occur in the presence of hCG (Jeppesen *et al.*, 2012; Rao *et al.*, 1977; Peng *et al.*, 1991), therefore providing a putative mechanism to why ovarian follicles did not respond to the ovulatory signal and why embryos derived were of poor quality (Bosch, 2010; Platteau *et al.*, 2004). This effect was not replicated by increasing the concentration of recombinant LH which has a shorter half life, implying the long half life of hCG may be responsible. hCG takes 66 times longer than LH to be cleared from the peripheral circulation due to the modified extension of the β subunit (Huirne *et al.*, 2004). The evidence suggests a ceiling level for LH exists above which is detrimental for embryo development (Hillier, 1994). The long half life of hCG explains why at higher concentrations this ceiling is exceeded whereas the lower half life of LH, means it is quicker cleared from the circulation and remains within a previously theorised LH window (Tesarik and Mendoza, 2002).

Oocyte quality is known to directly impact embryo development (Bosch, 2010). Fragmentation of the oocyte was a phenomenon only found in the ovarian stimulation groups and has been shown previously not to be compatible with continued embryo development (Veeck, 1988). Fragmentation appeared to be worsened by the presence of LH, supporting previous clinical evidence which suggested that overstimulation with LH had negative effects on follicular atresia and luteinisation and consequently oocyte development in a dose dependant manner (Hillier, 1994). Cumulus cells were analysed for angiogenic factors, the presence of which has previously been correlated with increased retrieval rate of mature oocytes, improved fertilisation and embryo quality and consequently greater pregnancy rates (Nargund *et al.*, 1996; Bhal *et al.*, 1999; Coulam *et al.*, 1999; Huey *et al.*, 1999; Du *et al.*, 2006; Shrestha *et al.*, 2006; Chui *et al.*, 1997; Van Blerkom *et al.*, 1997; Borini *et al.*, 2005). Our results suggest angiogenesis may be reduced following ovarian stimulation based on reduced expression of *MYHII*, a factor which promotes angiogenesis by inducing synthesis of AngII which is associated with steroidogenesis, meiotic maturation and ovulation (Ferreira *et al.*, 2011; Siqueira *et al.*, 2012); therefore reduced expression may impair oocyte quality. Treatment with PMSG caused a greater decrease in *MYHII* which may explain why embryo development was more perturbed in this treatment group.

The genetic experiments in Chapter 5 attempted to address whether the observed reports of imprinting and methylation errors following ART in animal models (Market-Velker *et al.*, 2010; Menezo *et al.*, 2010; Zhang *et al.*, 2010; Mundim *et al.*, 2009) and human subjects (Strawn *et al.*, 2010; Katari *et al.*, 2009; Lawrence and Moley, 2008; Sato *et al.*, 2007) were altered by ovarian stimulation using different commercial preparations of exogenous gonadotrophins. In our study, ovarian stimulation with PMSG and 0.5IU hMG resulted in a decrease of *IGF2*, which modulates the expression and activity of nutrient transporters in the placenta (Constância *et al.*, 2002). Reduced *IGF2* gene expression has been associated with intra uterine growth retardation (IUGR) (Grandjean *et al.*, 2000) and Wilms tumour, a childhood cancer of the kidney, in

addition to other forms of benign and malignant cancers (Ravenel *et al.*, 2001; McCann *et al.*, 1996; McMinn *et al.*, 2006). Alterations of imprinted genes has previsouly been shown to result in impeded embryo development (Shi and Haaf, 2002), which was also apparent in the ovarian stimulation regimes in our study. Loss of *H19* expression was evident in blastocysts from the 5IU hMG and 0.5IU Pergoveris treatment groups, which had the highest levels of developmental arrest and the poorest blastocyst quality grades. This may be a consequence of insufficient maternal mRNA stores through acceleration of oocyte maturation, reducing the ability of the embryo to maintain imprints (Li *et al.*, 2010).

The expression of important genes in the RAS system were also analysed, which have been shown to play a pivitol role in decidualisation, implantation and placentation by regulating blood flow, oestradiol secretion and prostaglandin synthesis (Nielsen *et al.*, 2000). Disruptions in the RAS has been implicated in cases of pre-eclampsia and IUGR, specifically down regulation of AT_1 receptors in the placenta (Li *et al.*, 1998) therefore over-expression of AT_1 may cause overgrowth of the foetus (Tower *et al.*, 2010) and indeed progeny following ovarian stimulation had heavier body mass compared to *in vivo* controls. Furthermore all ovarian stimulation groups had lower expression levels of *VEGFA* which has been associated with deciduas formation during implantation (Douglas *et al.*, 2009; Plaisier *et al.*, 2007) potentially explaining why less of the transferred blastocysts in the ovarian stimulation groups resulted in live birth.

All experimental chapters in this thesis indicated ovarian stimulation may cause detrimental effects which could potentially affect the long term health of offspring born following ART. Therefore the embryo transfer studies in chapter 6 were performed to confirm or refute previous hypotheses that poor embryo development and altered gene expression would have adverse effects on pregnancy and live pups generated. In accord with previous reports, ovarian stimulation caused a decrease in the number of live pups born (Zhang *et al.*, 2010; Ertzeid and Storeng,

2001). In this study we used sterile mated control females as surrogates for all the treatment and control groups and transferred the same number of blastocysts to the recipients. This allowed us to control for any endometrial effects which could have resulted if ovarian stimulation was used and allowed us to only assess the effects of the embryo on implantation and progeny. Surprisingly, the human gonadotrophin preparations, despite having improved embryo development compared to PMSG, had reduced live pups per embryo transferred, which was exacerbated in stimulation groups such as Pergoveris where the blastocysts were more developmentally advanced. The results suggest there may be asynchronicity between the stage of embryos transferred and the endometrium receptivity.

Some authors have suggested infants conceived through ART have a higher incidence of childhood illness requiring healthcare utilisation, in particular for respiratory complaints (Koivurova *et al.*, 2003) and cardiovascular disease in later life (Yeung and Druschel, 2013). Our results showed ovarian stimulation caused alterations in internal organ relative body weight. The results of our study do indicate there may be some underlying developmental abnormalities for the adverse neo-natal risks some authors have attributed to superovulation, although how this affects the function of the organ was not assessed in this work. *In vitro* culture in our study appeared to be the main cause for the organ growth effects on resulting progeny. This suggests that altered *in vitro* culture conditions may be the initial cause for the alterations and ovarian stimulation a further catalyst.

8.2 Potential limitations of work conducted

The aim of this work was to assess the consequences of ovarian stimulation with different gonadotrophin preparations on oocyte development, embryo quality, gene expression and ultimately the health of offspring. We chose to conduct this work in the murine model as it is the most widely utilised analogue model for pre-implantation embryo

development (Taft, 2008). However, mice are poly-ovulaters that release 8-12 oocytes depending on the strain, therefore the observed responses may not be similar to mono-ovulatory species such as humans (Nagy *et al.*, 2003). In addition human patients undergoing ART have fertility issues that may potentially predispose their gametes and subsequent embryos to heightened sensitivity to the superovulation and manipulation procedures required in those processes (Gardner and Lane, 2005). However, this fact enables the assessment of whether complications associated with ART are a consequence of the procedures themselves or the infertility.

Throughout this study we used quantitative PCR to assess the effect of ovarian stimulation of gene expression both in the cumulus cells and embryo itself. However there are many post translational modifications required after synthesis of mRNA which makes it difficult to directly translate mRNA expression levels detected in this study to protein levels with biological activities (Greenbaum *et al.*, 2003). Furthermore only a selection of genes were assessed in each case therefore despite gene expression data correlating with expected phenotypic outcomes, the influence of alternative factors cannot be ruled out. The use of global gene expression arrays and an increase in repetitions will provide more evidence on how ovarian stimulation alters gene expression.

The analysis of embryonic gene expression in our study was always performed by trophectoderm biopsy of blastocyst hatching away from the ICM. This may have potentially dampened the negative consequences of ovarian stimulation on gene expression as embryos capable of developing to the blastocyst stage especially hatching are considered to have better developmental potential. Perturbations in gene expression have also been correlated with blastocyst morphology with hatching blastocysts shown to have the highest of proportion of normally methylated embryos (Fauque *et al.*, 2007). In our study only the mural trophectoderm cells, i.e. those furthest away from the ICM were analyzed. It is these cells which differentiate into primary trophoblast giant cells during implantation (Negrón-Pérez *et al.*, 2013), therefore we cannot definitively conclude that

the gene expression in this region is comparable with the rest of the blastocyst. The analysis of whole blastocysts may have provided different results due to the proposed differential gene expression between the ICM and TE (Ozawa *et al.*, 2012). The analysis of complete blastocysts would also enable the assessment of embryos at different developmental stages, which may have genetic pertubations and were not analysed in our study due to not meeting the requirements for biopsy.

8.3 Applications of this work

Ovarian stimulation regimes are tailored to individual patients based on their pre-treatment assessment and monitored throughout using ultrasound and serum blood concentration analyses (Jayaprakasan *et al.*, 2008). It is general practice that if a woman is under responding (e.g. has 3 or fewer follicles developing) (Ferraretti *et al.*, 2011), the dose of the exogenous gonadotrophin preparation in use is increased, in an attempt to recruit more follicles to develop. Our study indicated that increasing hMG high enough led to a reduction in the number of oocytes ovulated. What is significant is the severe retardation of embryo development and reduced blastocyst quality following high hMG treatment. We postulated based on previous studies which have shown that hCG leads to down regulation of the LH receptor on granulosa cells (Jeppesen *et al.*, 2012; Rao *et al.*, 1977; Peng *et al.*, 1991) that these consequences may have been due to disruption of ovulation and impaired steroidogenesis (Westergaard *et al.*, 2000; Fleming *et al.*, 2000). Contrary to our results, systematic reviews on clinical data indicates that the use of this preparation increases live birth rates by 3-4% (Hill *et al.*, 2012). The results in our study highlight the requirement for exogenous gonadotrophins to stay within the LH window, which may be different between mice and human patients, particularly if down regulation is used.

8.4 Future work

There were a few areas in our research where we relied on previous studies to confer observations with mechanistic actions. For example,

although it has been shown that hCG can lead to down regulation of the LH receptor on granulosa cells in human (Jeppesen *et al.*, 2012) and rats (Rao *et al.*, 1977; Peng *et al.*, 1991), it was not confirmed in our study. In addition we hypothesised that the reduced vascularity of ovarian follicles after superovulation inferred from cumulus cell analyses may have resulted in spindle defects (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997) explaining the inappropriate division of genetic material and failure to complete syngamy in developmentally arrested metaphase II zygotes from the ovarian stimulation regimes (Pesty *et al.*, 2007). This hypothesis could be confirmed by visualising the mitotic spindle in un-cleaved metaphase II oocytes by immuno-histochemical staining of the cytoskeletal structure through injection of fluoroscein-labelled α-tubulin (Paddy *et al.*, 1996). Finally, our results provided support for the theory that ovarian stimulation may result in disruption of maternal effect gene products which are required to maintain correct imprinting throughout embryo development (Li *et al.*, 2010; Rivera *et al.*, 2008). Potentially the analysis for altered gene expression of the oocyte specific Dnmt1o iso-form which has been demonstrated to maintain methylation imprints until the embryonic genome is activated would provide further evidence (Mertineit *et al.*, 1998).

Although our study indicated organ growth discrepancies in progeny following ovarian stimulation and *in vitro* culture, these conclusions were based solely on the weight of the organs made relative to the total body mass of the mouse. More in depth offspring analyses are required to generate more data and provide further support for the hypotheses made. For example, reduced heart mass and enlarged kidneys are indicators of hypertension and cardiovascular disease (Yeung and Druschel, 2013; Ziakas *et al.*, 2005; Päivänsalo *et al.*, 1998). Monitoring of blood pressure by telemetry using a digital recording device on the mouse's tail would provide long term undisturbed data readings for the developing offspring to monitor fluctuations in cardiac function throughout post natal development (Van Vliet *et al.*, 2006). Enlarged kidneys are also an indication of diabetes. The quantification of glucose and insulin in tail

bleeds of mouse progeny would provide further evidence into the adverse effects of ART on metabolic syndromes (Geutskens *et al.*, 2004). Finally our results showed reduced lung mass of ovarian stimulation progeny which may indicate a physical impairment of the lungs. Histological analysis of the lung tissue collected for alterations in bronchi tree structure or arteriole structure may provide some indications. Alternatively a lung function test can be performed on live mice to measure airway responsiveness (Glaab *et al.*, 2007). There are numerous methods but barometric whole body plethysmography is the easiest to perform whereby conscious mice are put into an enclosed chamber and pressure fluctuations during the breathing cycle are recorded. This method provides continual monitoring of lung function throughout development with mammal disturbance to the animal (Hamelmann *et al.*, 1997).

8.5 Final Summary

In summary, it has been observed throughout this thesis, that ovarian stimulation causes perturbed embryo development, gene expression alterations and postnatal growth aberrations. The observations of these adverse effects in a fertile mouse model, suggest underlying fertility pathology is not the main cause for adverse peri natal outcomes following ART. *In vitro* culture alone did appear to have an effect on progeny growth, suggesting suboptimal culture environment may be the cause for some of the observed consequences, which were worsened by ovarian stimulation. Comparison of human gonadotrophin preparations highlighted the complexity of effects resulting from the use of different doses of gonadotrophin preparations which vary in terms of their biological clearance rate, FSH isoforms and level of LH-activity. Of significant importance was the demonstration continuous administration of high levels of hCG impaired ovulation

and subsequent embryo development, which indicates caution should be taken not to exceed the LH window when delivering exogenous gonadotrophins. The main area for improvement of this work is to consolidate the correlations drawn between phenotypic observations and mechanistic biological process with further evidence, to provide more sound data on the adverse effects of ovarian stimulation.

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APPENDIX

Appendix 1: Media Compositions

A.1.1 G-MOPS

A handling medium designed to support manipulation of oocytes and embryos outside the incubator (ambient environment). It is supplemented with 5% HSA and warmed to 37°C prior to use.

Amino acids	Buffer	Antibiotics	Sugars	Ionic Compounds	Other
Alanine, Alanyl-glutamine, Aspargine, Aspartate, Glutamate, Glycine, Proline, Serine, Taurine	MOPS	Penicillin G	Sodium Lactate Sodium Pyruvate Low Glucose	$CaCl_2$ $MgSO_4$ KCl $NaHCO_3$ NaCl NaH_2PO_4	Water

Table A.1: The Components of G-MOPS

A.1.2 G-1™

Supports development of cleavage stage embryos from the pro-nucleate to 8-cell stage. It is equilibrated at 37°C, 6% CO_2 prior to use.

Amino acids	**Buffer**	**Antibiotics**	**Sugars**	**Ionic Compounds**	**Other**
Alanine, Alanyl-glutamine, Aspargine, Aspartate, Glutamate, Glycine, Methionine, Proline, Serine, Taurine	Bicarbonate	Gentamicin	Sodium Lactate Sodium Pyruvate Low Glucose	$CaCl_2$ $MgSO_4$ KCl $NaHCO_3$ NaCl NaH_2PO_4 Sodium Citrate	EDTA Hyaluronan Lipoic acid Water

Table A.2: The Components of G-1™

A.1.3 G-2™

Supports development of 8-cell stage embryo until the blastocyst stage. It is equilibrated at 37°C, 6% CO_2 prior to use.

Amino acids	Buffer	Antibiotics	Sugars	Ionic Compounds	Other
Alanine, Alanyl-glutamine, Arginine, Aspargine, Aspartate, Cystine, Glutamate, Glycine, Histidine, Isoleucine, Leucine, Lysine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, Valine	Bicarbonate	Gentamicin	Sodium Lactate Sodium Pyruvate Low Glucose	$CaCl_2$ KCl $NaHCO_3$ NaCl NaH_2PO_4 Sodium Citrate Sodium Pantothenate	Hyaluronan Pyridoxine Riboflavin Thiamine Water

Table A.3: The Components of G-2™

A.1.4 G-PGD

The calcium and magnesium free media facilitates embryo biopsy and maintains pH.

Amino acids	**Buffer**	**Antibiotics**	**Sugars**	**Ionic Compounds**	**Other**
Alanine, Alanyl-glutamine, Aspargine, Aspartate, Glutamate, Glycine, Proline, Serine	MOPS	Penicillin G	Glucose Sodium lactate Sodium pyruvate	KCl $NaHCO_3$ NaCl NaH_2PO_4	Water

Table A.4: The Components of G-PGD

Appendix 2: GMOPS- Good media or poor substitute

Initially oviducts were excised from mice subsequent to cervical dislocation and stored in centre wells filled with GMOPS in a non-gassed incubator until ready to collect one cell pro-nuclear embryos. Longer exposure times to GMOPS manifested in delayed embryonic growth as evidenced by degenerated single celled embryos on day 2 of development (Figure A.1). This morphological discrepancy was detectable after exposure times of just 15 minutes.

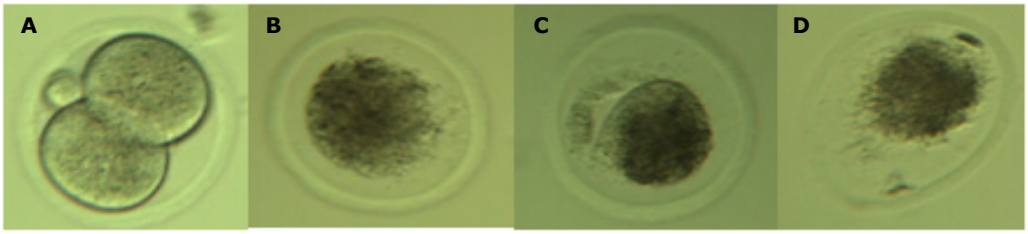

Figure A.1: Morphological microscopic photographs day 2 of development after initial exposure times to GMOPS for <1 **(A)**, 15 **(B)**, 30 **(C)** and 45 **(D)** minutes.

Prolonged exposure times resulted in a higher proportion of fragmented, degenerated and arrested one cell embryos, compared to the control where GMOPS exposure was less than 1 minute and achieved a 100% cleavage rate on day 2 of development. GMOPS exposure for 15, 30 and 45 minutes achieved cleavage rates of 66, 70, 25% respectively. Assessment of the nuclear maturity of the degenerating one cell embryos confirmed they were arrested fertilised embryos (Figure A.2).

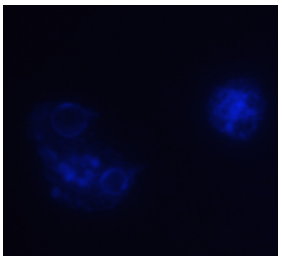

Figure A.2: DAPI stained one cell degenerated embryo exhibiting the presence of two pronuclei in the cytoplasm, indicating a fertilised status.

Perturbed embryonic development was documented throughout *in vitro* culture and was clearly evident at media change on day 3 (Figure A.3). Reduced proportions of the expected 4-8 cell and morulas in the GMOPS exposure groups compared to the control were clear with only 63%, 21% and 16% of embryos progressed to this stage in the 15 minute, 30 minute and 45 minute exposure groups respectively, compared to 98% in the control. Analysis of these proportional differences using the Fishers Exact test showed significant ($P<0.001$) differences as early as 15 minutes exposure to GMOPS.

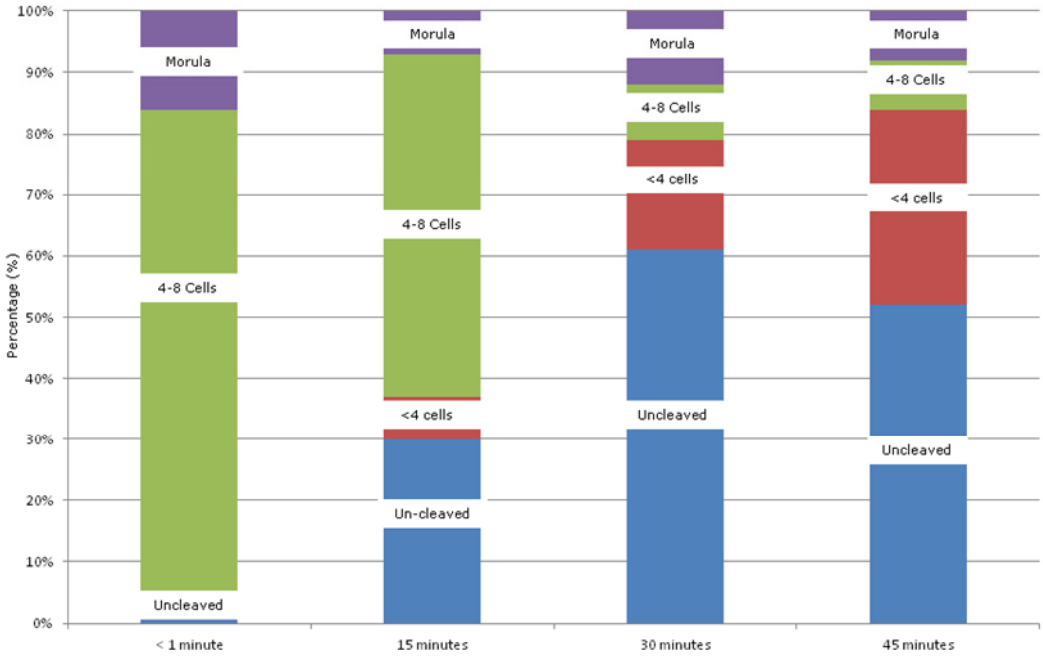

Figure A.3: Embryo development at day 3 of culture for control, 15 minute, 30 minute and 45 minute GMOPS exposure groups. All values are a percentage of zygotes collected and cultured *in vitro*.

The detrimental effect of GMOPS on development significantly impaired the ability of embryos to progress to the blastocyst stage (Figure A.4). Comparative blastocyst developmental rate (BDR) was observed between oviducts exposed to GMOPS for 30 minutes with the control ($P>0.05$). Exposure for 15 and 45 minutes demonstrated a significant decrease in BDR, $P=0.0042$ and $P=0.00002066$ respectively. There was no difference in the morphological appearance of viable blastocysts in each exposure group with expanded blastocysts observed in all groups on day 5 of

culture. The quantity of degenerated collapsed blastocysts was however exasperated in the groups of embryos exposed to GMOPS for longer than 1 minute, in a time dependent manner (Figure A.5).

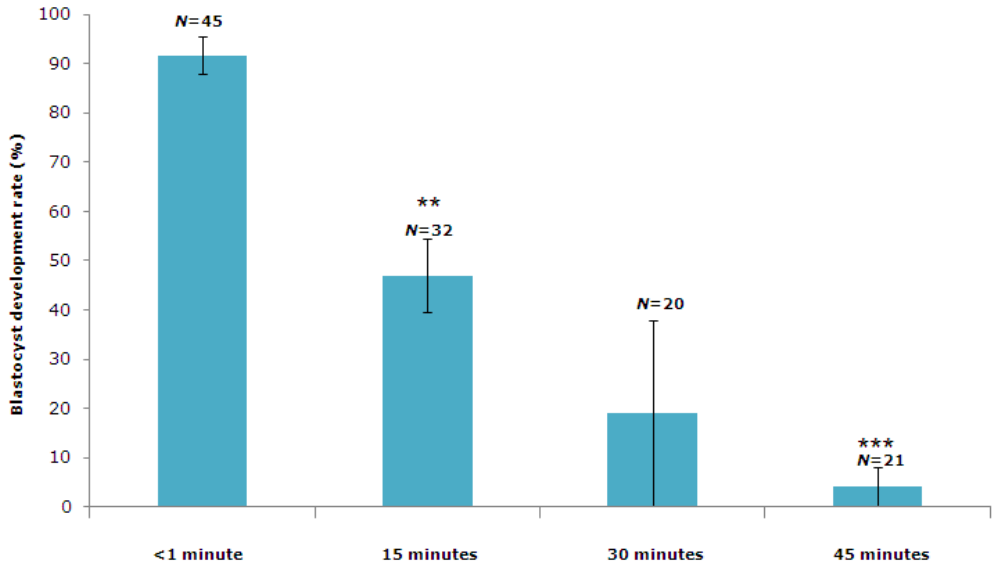

Figure A.4: Mean (%) blastocyst developmental rate (BDR) ± Standard error of the mean (SEM) for control, 15 minutes, 30 minutes and 45 minutes GMOPS exposure groups. *N*=total number of embryos t. **=*P*<0.01 and ***=*P*<0.001 compared with control.

Due to these observed deleterious effects observed with exposure to GMOPS the embryo collection protocols were refined so that exposure was as transient as possible. This involves the euthanasia of a single mouse whose embryos are then immediately harvested without being pooled in GMOPS until ready for collection.

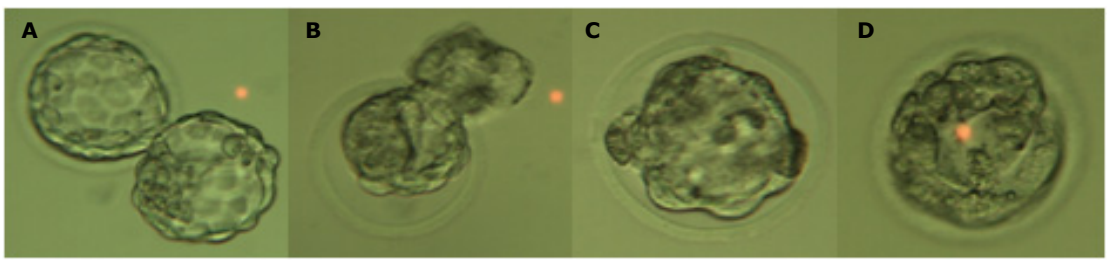

Figure A.5: Embryo morphology on day 5 after exposure of oviducts to GMOPS for <1 **(A)**, 15 **(B)**, 30 **(C)** and 45 **(D)** minutes.

Appendix 3: Daily monitoring of embryos does not impair development

Embryos were monitored daily for their developmental progress by taking them out of the incubator and assessing them under the microscope. This was not detrimental to embryo development as shown in Figure A.6, whereby the proportion of embryos continuing development to the blastocyst after un-disturbed *in vitro* culture (colour), was comparable to embryos removed daily from the incubator to monitor progress (greyscale, P>0.05). The proportion of embryos at each stage was inferred at the time of media change and blastocyst analysis for the un-disturbed group. This may explain why there is an un-significant reduction in the number of embryos developing for some cohorts, as the cleavage status of degenerated embryos would not have been available.

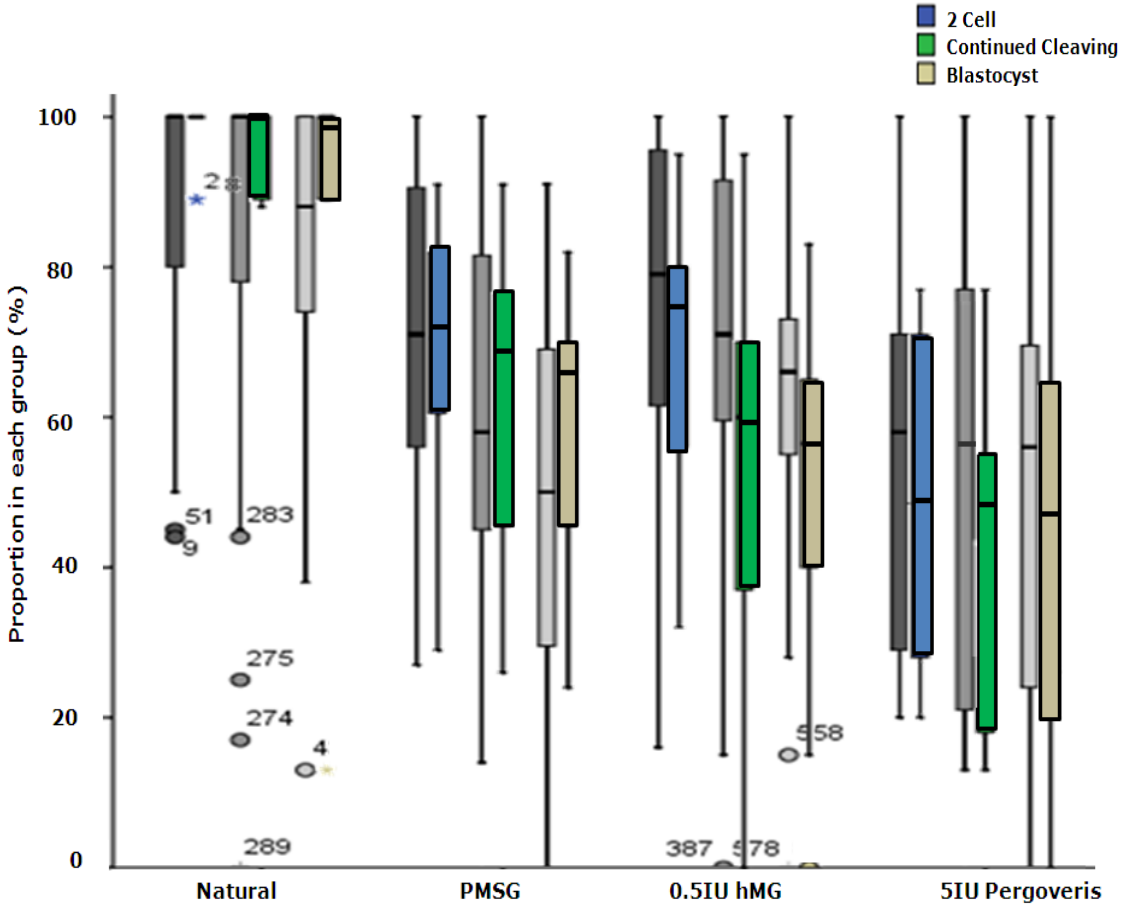

Figure A.6: Proportion of embryos at each developmental stage with (greyscale) or without (colour) daily monitoring outside the incubator.

Appendix 4: Endogenous control selection for PCR by geNorm

The geNorm reference gene selection kit by Primer design was used to select the most stable constitutively expressed reference (housekeeping) gene for our experimental conditions, to normalise the quantitative real time experiments. A panel of 6 genes were selected to assess the ideal reference gene for our experimental study to increase the accuracy and sensitivity of our PCR experiments. Using the geNorm software qBase^{PLUS} by Biogazelle, the genes were ranked according to their expression stability.

Both cumulus cell and trophectoderm biopsy cDNA samples were assessed with a panel of 6 genes:

- 18s ribosomal RNA (18s)
- eukaryotic translation initiation factor 4A2 (EIF4A2)
- succinate dehydrogenase complex, subunit A (SDHA)
- glyceraldehyde-3-phosphate dehydrogenase (GADPH)
- ATP synthase subunit (ATP5B)
- Actin beta cytoplasmic (ACTB)

Each 10µl reaction was composed of 0.5µl lyophilised primer and probe mix, 5µl of fast advance master mix, 2µl nuclease free water and 2.5µl of the cDNA sample. The plates were run at the following cycling conditions for 50 cycles: 10 minutes at 95°C, 15 seconds at 95°C, 30 seconds at 50°C and 15 seconds for 72°C. The data file was then exported to qBase^{PLUS} software for analysis.

The software generates graphs depicting average expression stability (M) of reference genes with the least stable gene at the left leading to the

most stable gene on the right. An M value less than 1 shows suitability of the reference gene for use in the experimental conditions. All reference genes were less than 1 for the cumulus cell samples (Figure A.7A), however none of them were for the biopsy cell samples (Figure A.7B). This can be explained by the low and variable sample sizes for this experiment.

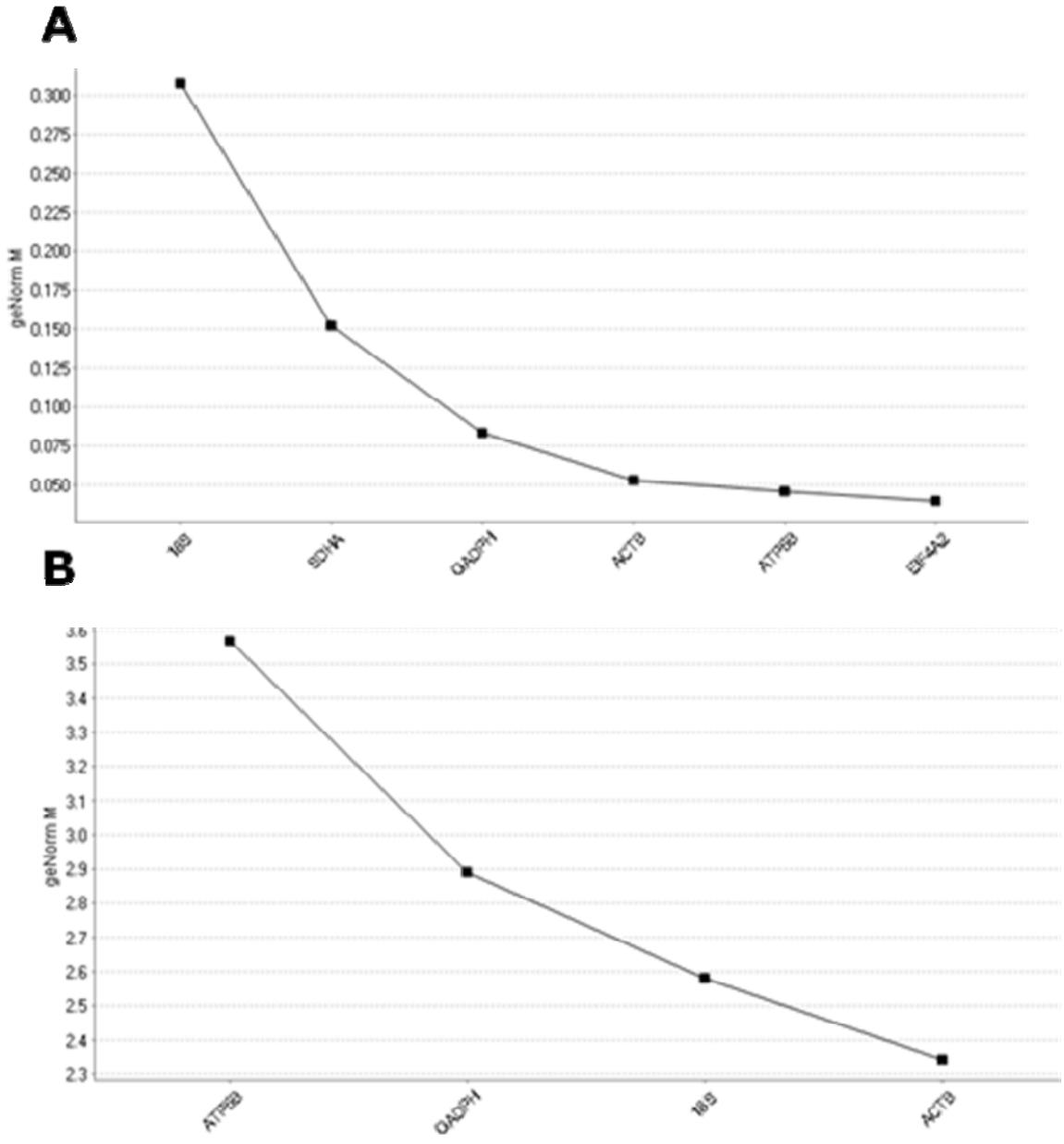

Figure A.7: qBase^{PLUS} average expression stability curves for **(A)** cumulus cell samples and **(B)** trophectoderm biopsy cell samples.

A combined analysis of the reference genes to function equally well in both experimental conditions ranked ACTB as the most suitable, closely followed by 18s (Figure A.8). 18s was chosen as the endogenous control for our PCR experiments based on its stability and also its earlier detection (CT value) at approximately the 25th PCR cycle as opposed to the 33rd for ACTB in the biopsy cell samples. It was hypothesised therefore that 18s would be more capable of detecting the lower starting differences for this cell sample.

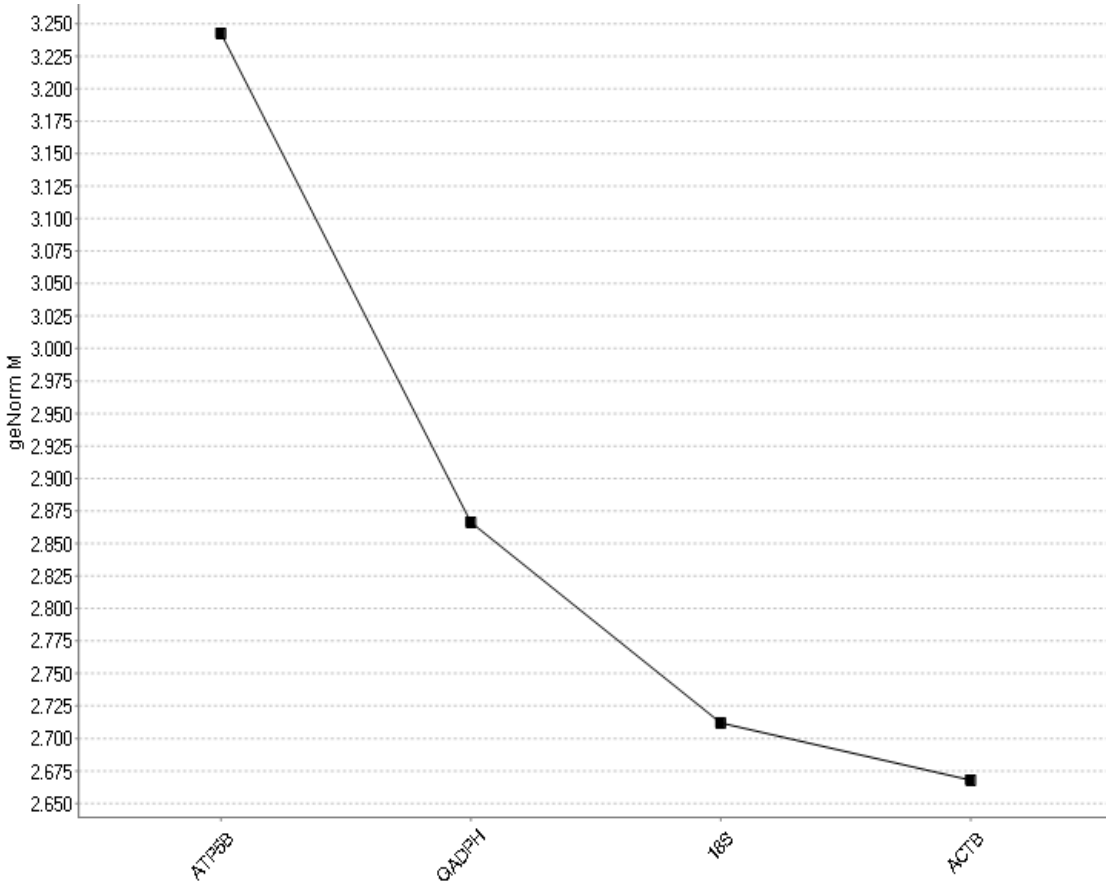

Figure A.8: qBase^{PLUS} average expression stability curve for combined analysis of cumulus cell and trophectoderm biopsy cell samples.

Appendix 5: In vivo blastocyst control method

Five to six week old B6CBAF1 female mice were superovulated with the 0.5IU hMG protocol and remained in the animal house until developmental day 4 to collect *in vivo* blastocysts to be used as standards and calibrator in the PCR experiments. Blastocysts were flushed from the uterine horns of the mouse using a 5ml syringe with a 30 Gauge needle containing warmed G-MOPS^{TM} medium (Figure A.9).

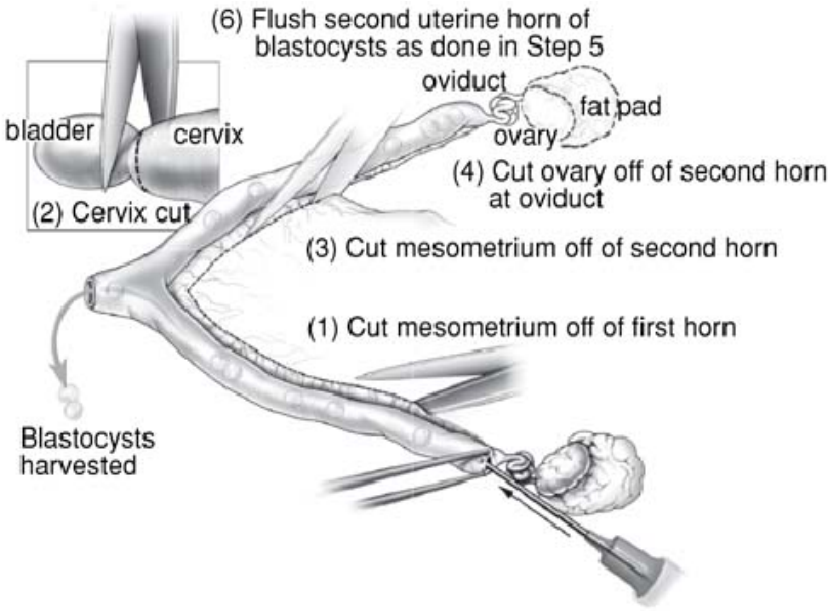

Figure A.9: 30-G needle inserted inside oviduct and blastocysts flushed out of uterus through cervical opening by injection of G-MOPS^{TM} medium. (Sakai and Onodera, 2008).

Appendix 6: Embryo development for treatment with 2.5IU rFSH in mature mice

Ovarian stimulation with rFSH in immature mice did not yield any blastocysts. An alternative research group reported a 72% blastocyst rate in mature mice of the same strain (Edwards *et al.*, 2005). Therefore we repeated the 2.5IU rFSH treatment dose in 8 week old females to assess whether the use of immature mice were the reason for embryo developmental arrest in our study. Blastocysts still did not develop however, indicating an alternative reason is behind the failure to reach blastocysts when rFSH alone is used for ovarian stimulation.

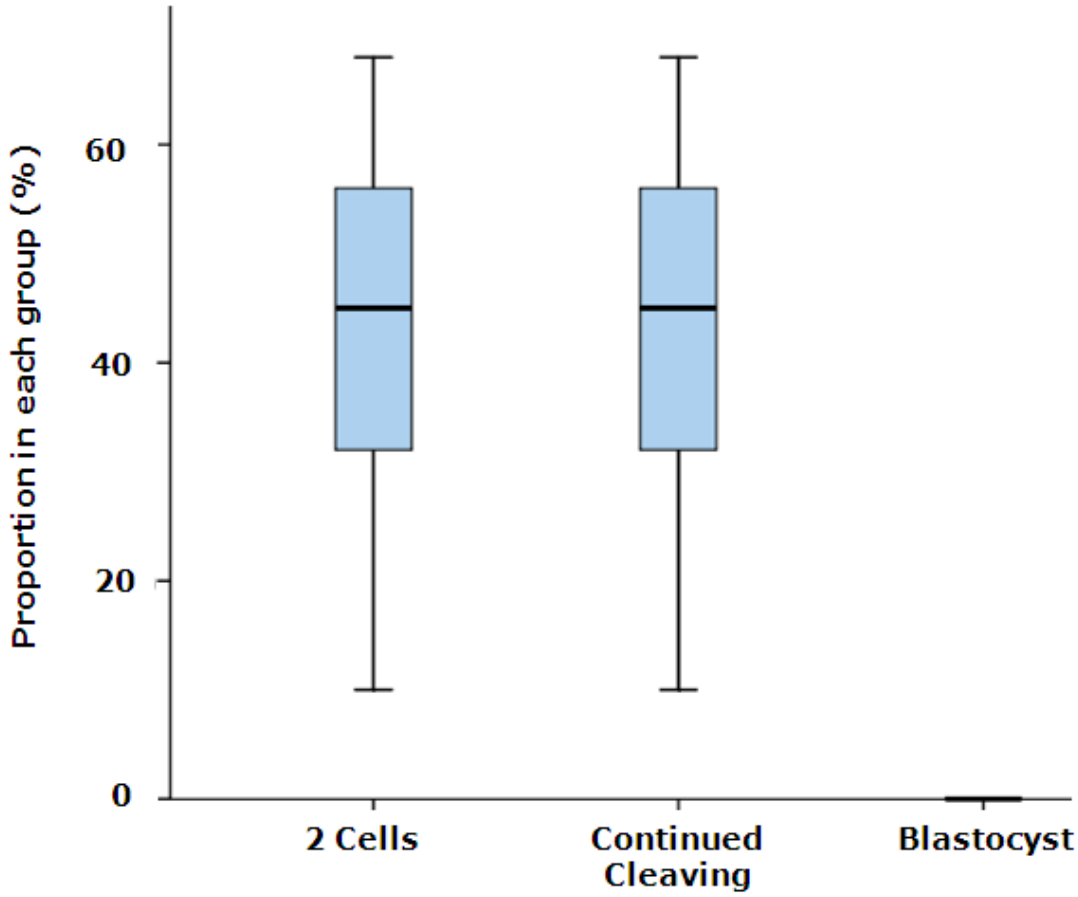

Figure A.10: Box plot showing the median proportion of embryos cleaving to the 2 cell stage, continuing cleavage past this stage and reaching the blastocyst stage for the 8 week old female 2.5IU rFSH treatment groups relative to number of zygotes collected. Bars represent the inter-quartile range.

Appendix 7: Ovarian histology methods

Collection and fixation of ovarian tissue

Ovaries were collected simultaneously to zygotes and fixed in Bouin's solution [(made in house; 25ml of 37% Formaldehyde solution (F1635, Sigma-Aldrich), 75ml of 1.3% Picric acid solution (P6744, Sigma-Aldrich) and 5ml glacial Acetic acid (A/0400/PB17, Fisher Scientific, Leicestershire, UK)] for 24 hours and stored in 70% alcohol until ready to be processed using a standardised tissue processing protocol.

Paraffin wax embedding

Fixed ovaries were dehydrated with a graded series of ethanol (70%, 90% and 95% alcohol for 60 minutes in each concentration, and 100% alcohol 3X, 60 minutes each change) (458600, Aldrich Chemistry), cleared with xylene treatment (4X, 60 minutes each change) (X/0250/PB17, Fisher Scientific) and embedded in molten paraffin wax (3X, 1 hour 20 minutes each change) in an automatic tissue processor (Shandon Excelsior, Thermo Scientific, ELECTRON CORPORATION).

Mounting of sections

Once the tissue was embedded in paraffin blocks (Tissue Embedder, TISSUE-TEK III), 5µm thick sections were cut using a rotary microtome (Leitz, 1512). Every tenth section was floated on a plate with 50% IMS on its surface before being added to a heated water bath set at 45°C (Paraffin Section Mounting Bath, MH8515, Barnstead Electrothermal). Multiple sections were subsequently captured by a glass slide (Superfrost® Plus, Thermo Scientific). After air drying at room temperature for at least 1 hour on a slide drying bench, the slides were baked for a minimum of 16 hours in an oven set at 50°C (Genlab, Widnes England).

Haematoxylin and Eosin staining

After baking, the sections were deparaffinised by incubation in xylene for 5 and 2 minutes followed by ethanol (100% ethanol, 2X for 2 minutes, 90% ethanol, once for 2 minutes and 70% ethanol once for 2 minutes) and rehydrated with distilled water (once for 5 minutes). Subsequently, sections were stained with haematoxylin by placing the slide holder in Harris' haematoxylin (3519455S, BDH) for 5 minutes, rinsing with tap water until the water ran clear and dipping three times in acid alcohol [10ml concentrated hydrochloric acid (HCl, 37%, H/1200PB17, Fisher Scientific) was added to 1 litre 70% ethanol]. The stain was developed for 5 minutes in Scott's tap water [3.5g sodium bicarbonate (S-8875, Sigma) and 20g magnesium sulphate (M/1050/53, Fisher Chemicals, Leicestershire, UK) added to 1 litre of distilled water] and rinsed again in tap water before eosin staining. The slide holder was added to 0.1% Eosin yellowish (341972Q, BDH) for 5 minutes before being washed in tap water until the water ran clear. This was followed by dehydration in 70% ethanol for 2 dips, 90% ethanol for 2 minutes and 100% ethanol 2X for 2 minutes. Finally, slides were placed in xylene 2X for 2 minutes and cover slipped with DPX mounting medium (D/5319/05, Fisher Scientific). After overnight drying, the total number of surface CLs for each mouse ovary was quantified using bright field light microscopic analysis (Nikon Eclipse 90i, Nikon Instrument Inc, Derby, UK).